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THE BIOCHEMICAL JOURNAL

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Trypsin-Catalysed Transpeptidations

By S. G. WALEY* and J. WATSON
Courtaulds Ltd., Research Laboratory, Maidenhead, Berkshire

(*Received* 8 *December* 1953)

The way in which proteins are formed in nature is so far obscure. It is generally considered that transpeptidation reactions may play a part in the process (Borsook, 1952; Campbell & Work, 1953), and recently a variety of enzymes have been found to catalyse transamidations. The transamidation reaction may be written:

$$XNH.CHR.CO{+}NHY + Z.NH_2 \rightarrow XNH.CHR.CO.NHZ + Y.NH_2.$$

Thus in an early example (Bergmann & Fraenkel-Conrat, 1937), $X = C_6H_5.CO$, $R = Y = H$, $Z = C_6H_5$; many of the reactions studied recently by Fruton and his co-workers have also been of this type with the acceptor molecule ($Z.NH_2$) an amino acid or peptide, so that the reaction leads to the lengthening of a peptide chain (Fruton, Johnston & Fried, 1951; Jones, Hearn, Fried & Fruton, 1952). The same molecule may serve as both donor and acceptor when a dipeptide amide is used, leading to polymer formation; thus glycyl-L-phenylalanine amide polymerized in the presence of cathepsin-C (Jones *et al.* 1952). True peptides (as opposed to amides) can also undergo transpeptidation; this has been especially studied with γ-glutamyl peptides (Hanes, Hird & Isherwood, 1952; Kinoshita & Ball, 1953; Fodor, Miller & Waelsch, 1953).

Little, however, is known of the scope of transpeptidation reactions, nor is it known whether they are to be expected from the action of proteolytic enzymes on peptides. We have examined the transferase activity of a typical, crystalline, proteolytic enzyme, trypsin; both true peptides and amides have been used as substrates. The formation of dilysine by the action of a mixture of trypsin and chymotrypsin on the tripeptides lysyltyrosyllysine and lysyltyrosylleucine was reported earlier (Waley & Watson, 1951). It has now been found that trypsin alone will effect this transpeptidation and also catalyses transpeptidations with the following substrates: dilysine, lysine amide, lysyllysyltyrosine amide and pentalysine.

Several proteolytic enzymes have recently been found to catalyse the hydrolysis of esters which are related to their usual substrates (Schwert, Neurath, Kaufman & Snoke, 1948) and condensation reactions (analogous to transpeptidations) of the type

$$XNH.CHR.CO_2R' + Z.NH_2 \rightarrow XNH.CHR.CO.NHZ + R'OH$$

might therefore be expected. Brenner, Müller & Pfister (1950) found that, in the presence of chymotrypsin, esters of methionine (and some other amino acids) gave the di- and tri-peptides, some higher polymers, and also the amino acid itself. We have observed that trypsin catalyses both the polymerization of lysyllysine ethyl ester and its reaction with tyrosine amide, phenylalanine amide, tyrosyltyrosine amide and tyrosylleucine.

As in our earlier work (Waley & Watson, 1953*a*), the mixed anhydride method of Wieland & Sehring (1950) was much used for the synthesis of peptides. Recently, however, Vaughan (1952) has shown that racemization may accompany peptide synthesis when the mixed anhydride of an acylated dipeptide (e.g. I) is used; the extent of racemization depended

$$C_6H_5.CH_2O.CO.NH.CH_2.CO.NH.CH(CH_2.C_6H_5).CO.O.CO.O.CH_2.CH(CH_3)_2$$

(I)

greatly on the solvent. No racemization was observed when the mixed anhydride derived from an acylated amino acid was used. Now a tripeptide may be prepared either by coupling a protected dipeptide with an amino acid ester or by coupling a protected amino acid with a dipeptide ester. The latter method has been used to minimize the danger of racemization. We wished also to avoid the saponification of benzyloxycarbonyl peptide esters, which may be attended by side reactions (Waley & Watson, 1953*a*), and so used benzyl esters extensively. It was convenient in some cases to use formyl as a protective group, since it may be removed without splitting peptide bonds or benzyl esters (Waley, 1953). The azide method of peptide synthesis was also employed and the two routes used for the synthesis of lysyltyrosylleucine are shown below.

Similarly, two routes were used for the preparation of lysyltyrosyllysine; there was little to choose between the two methods for the preparation of the tripeptides, but the mixed anhydride method is simpler for the preparation of dipeptides.

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(1) $HCO.NH.CHR''.CO.O.CO.C_6H_5 + NH_2.CHR'''.CO_2.CH_2.C_6H_5 \rightarrow HCO.NH.CHR''.CO.NH.CHR'''.CO_2.CH_2.C_6H_5$

$\xrightarrow{Z.NH.CHR.CO.O.CO.C_6H_5} NH_2.CHR'.CO.NH.CHR'''.CO_2.CH_2.C_6H_5$ (II)

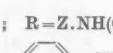
$\xrightarrow{H_2} Z.NH.CHR.CO.NH.CHR'.CO.NH.CHR'''.CO_2.CH_2.C_6H_5$ (III)

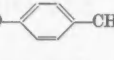
$\rightarrow NH_2.[CH_2]_4.CH(NH_2).CO.NH.CHR'.CO.NH.CHR'''.CO_2H$

(2) $Z.NH.CHR.CO.O.CO.C_6H_5 + NH_2.CHR'.CO_2.CH_3 \rightarrow Z.NH.CHR.CO.NH.CHR'.CO_2.CH_3$

$\xrightarrow{N_2H_4} Z.NH.CHR.CO.NH.CHR'.CO.NH.NH_2 \rightarrow Z.NH.CHR.CO.NH.CHR'.CON_3$

$\xrightarrow{NH_2.CHR'''.CO_2.CH_2.C_6H_5}$ (III)

($Z = C_6H_5.CH_2.O.CO.$; $R = Z.NH(CH_2)_4.$; $R' = HO$–  –$CH_2.$;

$R'' = CH_3.CO.O$–  –$CH_2.$; $R''' = (CH_3)_2{:}CH.CH_2.$)

One dipeptide, tyrosylleucine, was also prepared by two methods; the first was hydrogenation of the benzyl ester (II) and the second was hydrolysis of *O*-acetyltyrosylleucine methyl ester (from reduction of *O* - acetyl - *N* - benzyloxycarbonyltyrosylleucine methyl ester). The alkaline hydrolysis of peptide esters is completed in a few minutes in 0·1 N alkali, so that there is usually little danger of racemization. The esters of lysine peptides, however, were shown chromatographically to give by-products in this reaction.

EXPERIMENTAL

Materials

General. The values of the p*K* of the α-amino group of the peptides and their α-amino N as a fraction of the total N were determined by electrometric titration in 0·083 M-KCl at 25·8° (Waley & Watson, 1953*a*). The microanalyses were carried out by Mr G. Ingram of these laboratories. The preparation of the peptides of lysine has been described already (Waley & Watson, 1953*a*). Crystalline trypsin (50% $MgSO_4$, $7H_2O$; Armour Laboratories, Lindsey Street, London, E.C. 1) was used. L-Amino acids were used throughout. The error of the optical rotations is the standard deviation, calculated according to the method of Dean & Dixon (1951) for a small number (about ten) of observations. All the peptides prepared were chromatographically homogeneous in the solvent employed (p. 532). The m.p.'s are uncorrected.

Lysine amide. ε-Benzyloxycarbonyllysine amide hydrochloride (Bergmann, Zervas & Ross, 1935) was hydrogenated in aqueous methanol containing one mol. prop. of HCl, using Pd black as catalyst. *Lysine amide dihydrochloride*, crystallized from methanol, had m.p. 217–218° (decomp.). (Found: N, 18·5; Cl, 32·3. $C_6H_{15}ON_3$, 2HCl requires N, 19·3; Cl, 32·6%.)

Dibenzyloxycarbonyllysyltyrosine amide. The peptide synthesis of Wieland & Sehring (1950) was used. Benzoyl chloride (0·58 ml.) was added to dibenzyloxycarbonyllysine (Bergmann *et al.* 1935) (2·07 g.) and *N*-ethylpiperidine (0·67 ml.) in tetrahydrofuran (5 ml.) at 0°. After 2 min. the mixture was added to tyrosine amide (Blau & Waley, 1954) (0·9 g.) suspended in ethyl acetate (10 ml.) and 1·24 N-$KHCO_3$ (6 ml.). The mixture was stirred for 40 min. and the *product* collected and recrystallized from methanol; yield 2 g. (70%); m.p. 183–184°. (Found: C, 65·0; H, 6·5; N, 9·8. $C_{31}H_{36}O_7N_4$ requires C, 64·6; H, 6·25; N, 9·7%.)

Lysyltyrosine amide dihydrochloride. Dibenzyloxycarbonyllysyltyrosine amide was reduced by the method described for the preparation of lysine amide, except that 2 mol. prop. of HCl were used. The *product* crystallized on trituration under acetone; m.p. 250–253° (decomp.). (Found: Cl, 17·9; $C_{15}H_{24}O_3N_4$, 2HCl, H_2O requires Cl, 17·8%.)

Dibenzyloxycarbonyllysyl - ε - N - benzyloxycarbonyllysyltyrosine amide. Dibenzyloxycarbonyllysyl-ε-*N*-benzyloxycarbonyllysine was condensed with tyrosine amide, using the procedure described above. The *product* was recrystallized from methanol (yield 58%); m.p. 188°. (Found: C, 64·3; H, 6·4; N, 9·7. $C_{45}H_{54}O_{10}N_6$ requires C, 64·4; H, 6·4; N, 10·0%.)

Lysyllysyltyrosine amide trihydrochloride. Dibenzyloxycarbonyllysyl-ε-*N*-benzyloxycarbonyllysyltyrosine amide was hydrogenated in the presence of 3 mol. prop. of HCl, using 5% Pd—$BaSO_4$ as catalyst. The *product* crystallized under ethanol, and was recrystallized from methanol: ethanol: ether. (Found: C, 45·3; H, 7·7; Cl, 19·0. $C_{31}H_{36}O_4N_6$, 3HCl, H_2O requires C, 44·7; H, 7·3; Cl, 18·9%.)

N-Formyltyrosine. This was prepared by a modification of the methods of formylation of du Vigneaud, Dorfmann & Loring (1932) and Bäilmann, Jensen & Jensen (1934). Acetic anhydride (280 ml.) was added to tyrosine (90 g.) in 98–100% formic acid (840 ml.) and the solution kept at 35° for 30 min. After this time the ninhydrin test was faint (a portion of the solution was brought to pH 5 by diluting with aqueous sodium acetate and heated with aqueous ninhydrin). Water (90 ml.) was added, the solution evaporated under reduced pressure, and the residue recrystallized from 400 ml. of water (charcoal). *N*-Formyltyrosine hydrate (89 g., 79%) had $[\alpha]_D^{20} + 84·2 \pm 0·2°$ in ethanol (*c*, 2·6; based on anhydrous material). Fischer (1907) gives $[\alpha]_D^{20} + 84·9°$.

Optically pure tyrosine methyl ester was obtained in 68% yield by the action of methanolic HCl on *N*-formyltyrosine.

O-Acetyl-N-formyltyrosine. Acetic anhydride (25 ml.) was added to *N*-formyltyrosine hydrate (50 g.) in 1·175 N-NaOH (425 ml.) at 0°. After 10 min. the solution was acidified and the *acetyl compound* isolated by extraction with ethyl acetate and recrystallized from the same solvent (35 g., 63%), m.p. 163·5–164·5° (decomp.); $[\alpha]_D^{19} + 73·5 \pm 0·3°$ in ethanol (*c*, 1·9). (Found: C, 57·6; H, 5·3; N, 6·0. $C_{12}H_{13}O_5N$ requires C, 57·4; H, 5·2; N, 5·6%.)

O - *Acetyl* - N - *formyltyrosyl* - ε - *benzyloxycarbonyllysine benzyl ester.* *O*-Acetyl-*N*-formyltyrosine was condensed with ε-benzyloxycarbonyllysine benzyl ester (Erlanger & Brand, 1951) in the usual way. After recrystallization from ethyl acetate and then from 75% (v/v) aqueous ethanol the *product* was obtained in 48% yield, m.p. 157°. (Found: C, 65·5; H, 6·3; N, 7·3. $C_{33}H_{37}O_8N_3$ requires C, 65·6; H, 6·2; N, 7·0%.)

Tyrosyl-ε-benzyloxycarbonyllysine benzyl ester. Acetyl chloride (2·7 ml.) in dry benzyl alcohol (20 ml.) was added to *O* - acetyl - *N* - formyltyrosyl - ε - benzyloxycarbonyllysine benzyl ester (7·5 g.) in benzyl alcohol (47 ml.). After 40 hr., the *hydrochloride* was precipitated with dry ether (500 ml.), digested with ethyl methyl ketone (25 ml.), and recrystallized from water (20 ml.) containing 0·5 N-HCl (1 ml.); yield 5·1 g. (67%), m.p. 158·5–160°. (Found: C, 63·5; H, 6·3; N, 7·2. $C_{30}H_{35}O_6N_3$, HCl requires C, 63·2; H, 6·3; N, 7·4%.) Treatment of the hydrochloride with aqueous $NaHCO_3$ gave the *free base*, which crystallized from aqueous ethanol, m.p. 119–120°. (Found: C, 68·0; H, 6·8; N, 7·4; equiv. wt. 543. $C_{30}H_{35}O_6N_3$ requires C, 67·7; H, 6·4; N, 7·9%; equiv. wt. 532.)

Dibenzyloxycarbonyllysyltyrosyl- ε-benzyloxycarbonyllysine benzyl ester. (*a*) Dibenzyloxycarbonyllysine was condensed with tyrosyl-ε-benzyloxycarbonyllysine benzyl ester to give the *tripeptide ester*, which, after recrystallization from 75% (v/v) aqueous ethanol, and then ethyl acetate, had m.p. 128·5–129·5° (yield 55%). The analytical sample melted at 131–132°. (Found: C, 67·3; H, 6·5; N, 7·6. $C_{52}H_{59}O_{11}N_5$ requires C, 67·1; H, 6·4; N, 7·5%.)

(*b*) Condensation of dibenzyloxycarbonyllysine with tyrosine methyl ester by the method of Wieland & Sehring (1950) gave *dibenzyloxycarbonyllysyltyrosine methyl ester* (73%), which, after recrystallization from ethyl acetate: light petroleum (b.p. 60–80°) had m.p. 131–133°. (Found: C, 64·9; H, 6·6; N, 7·15. $C_{32}H_{37}O_8N_3$ requires C, 65·0; H, 6·3; N, 7·1%.) After keeping the methyl ester (13 g.) in methanol (50 ml.) containing hydrazine hydrate (10 ml.) for 24 hr., the product which had separated was washed with methanol and water and then digested with hot methanol. *Dibenzyloxycarbonyllysyltyrosine hydrazide* (90%) had m.p. 220–221°. (Found: C, 62·6; H, 6·4; N, 11·8. $C_{31}H_{37}O_7N_5$ requires C, 63·0; H, 6·3; N, 11·85%.) The hydrazide (5·9 g.) in acetic acid (25 ml.) and 2 N-HCl (5 ml.) at 0° was treated with $NaNO_2$ (1 g.) in water (3 ml.). The mixture was diluted with ice-water and the azide extracted with cooled ethyl acetate. The ethyl acetate solution was washed with cooled aqueous $KHCO_3$, dried (Na_2SO_4) and added to ε-benzyloxycarbonyllysine benzyl ester (from 4·1 g. of the hydrochloride) in ethyl acetate; the solution was diluted with an equal volume of ether and kept at 0° overnight. After filtration, the mixture was washed with 0·1 N-HCl, aqueous $KHCO_3$ and aqueous NaCl, dried (Na_2SO_4) and the solvents were removed by distillation. The residue was triturated under ether and recrystallized from 80% (v/v) aqueous methanol; yield 2·7 g. (29%), m.p. 128–130°. Mixed m.p. with tripeptide ester prepared by method (*a*), 130–132°.

Lysyltyrosyllysine dihydrochloride. Dibenzyloxycarbonyllysyltyrosyl-ε-benzyloxycarbonyllysine benzyl ester was hydrogenated in acetic acid in the presence of 2 mol. prop. of HCl, using Pd black as catalyst. The *tripeptide dihydrochloride* crystallized under ethanol and was precipitated from 90% (v/v) aqueous methanol by ethanol and ether in 40% yield. The material was dried *in vacuo* at 60° for analysis. (Found: C, 49·6; H, 7·2; N, 13·4; Cl, 14·0; α-amino N/total N, 20. $C_{21}H_{35}O_5N_5$, 2HCl requires C, 49·3; H, 7·3; N, 13·7; Cl, 13·9; α-amino N/total N, 20·0%); $[\alpha]_D^{20} = +22{\cdot}4 \pm 0{\cdot}6°$ in 0·5 N-HCl (*c*, 1·58); p*K* 7·25.

Tyrosyllysine. Hydrogenation in 75% (v/v) aqueous acetic acid of tyrosyl-ε-benzyloxycarbonyllysine benzyl ester hydrochloride (using 10% Pd-charcoal as catalyst) gave the peptide; satisfactory analytical values were not obtained, but the material was chromatographically homogeneous.

Leucine benzyl ester hydrochloride was prepared by the method of Miller & Waelsch (1952); later, this method was simplified by using the *benzenesulphonate* directly: it was recrystallized from water, and had m.p. 171°. (Found: C, 60·4; H, 6·7; N, 3·8. $C_{13}H_{19}O_2N$, $C_6H_6O_3S$ requires C, 60·2; H, 6·6; N, 3·7%.)

O-Acetyl-N-formyltyrosylleucine benzyl ester. *O*-Acetyl-*N*-formyltyrosine was condensed with leucine benzyl ester. The reaction mixture was extracted with ethyl acetate and the extracts were washed with 1·2 N-$KHCO_3$, 0·1 N-HCl and finally with water. The dried solution was concentrated and the *product* collected (yield 61%). After recrystallization from ethyl acetate, it had m.p. 156–157°. (Found: C, 66·1; H, 6·6; N, 6·35. $C_{25}H_{30}O_6N_2$ requires C, 66·05; H, 6·7; N, 6·15%.)

Tyrosylleucine benzyl ester hydrochloride. Acetyl chloride (2·8 ml.) in benzyl alcohol (14 ml.) was added to *O*-acetyl-*N*-formyltyrosylleucine benzyl ester (7 g.) in benzyl alcohol (20 ml.). After 48 hr., the *product* was precipitated with ether:light petroleum (b.p. 60–80°) (2:3), digested with ethyl acetate (27 ml.), and recrystallized from ethyl methyl ketone:$CHCl_3$, giving 3·6 g. (56%) of colourless needles, m.p. 169–172°; $[\alpha]_D^{19} - 16{\cdot}7 \pm 0{\cdot}3°$ in ethanol (*c*, 1·6). (Found: C, 63·0; H, 6·7; N, 6·6. $C_{22}H_{28}O_4N_2$, HCl requires C, 62·8; H, 6·9; N, 6·65%.)

Dibenzyloxycarbonyllysyltyrosylleucine benzyl ester. (*a*) Dibenzyloxycarbonyllysine was condensed with tyrosylleucine benzyl ester; the *product* was isolated by the method described under *O*-acetyl-*N*-formyltyrosylleucine benzyl ester. After recrystallization from ethyl acetate: light petroleum (b.p. 60–80°), it melted at 130·5–132° (yield 65%). (Found: C, 67·9; H, 6·7. $C_{44}H_{52}O_9N_4$ requires C, 67·7; H, 6·7%.)

(*b*) Dibenzyloxycarbonyllysyltyrosine hydrazide was converted into the azide and condensed with leucine benzyl ester, using the procedure described above; the product was obtained in 56% yield, m.p. 126–128°. Mixed m.p. with sample prepared by method (*a*), 127–129°.

Lysyltyrosylleucine dihydrochloride. Dibenzyloxycarbonyllysyltyrosylleucine benzyl ester was hydrogenated in acetic acid containing 3 mol. prop. of 0·5 N-HCl, using Pd black as catalyst. Evaporation of the solvent gave the crystalline *tripeptide dihydrochloride monohydrate*, which was recrystallized from methanol:acetone; p*K* 7·32. (Found: C, 49·1; H, 7·2; Cl, 13·6; α-amino N/total N, 26. $C_{21}H_{34}O_5N_4$, 2HCl, H_2O requires C, 49·1; H, 7·4; Cl, 13·8; α-amino N/total N, 25·0%.)

Tyrosylleucine. (*a*) Tyrosylleucine benzyl ester hydrochloride was treated with aqueous $NaHCO_3$ and the free base isolated by extraction with ethyl acetate. Hydrogenation in acetic acid using 10% Pd-charcoal gave the *dipeptide hydrate*, which crystallized from 80% (v/v) aqueous ethanol in colourless needles, m.p. 271–275° (decomp.); $[\alpha]_D^{21} + 6{\cdot}3 \pm 0{\cdot}4°$ in 0·5 N-HCl (*c*, 3·2). (Found:

C, 57·4; H, 7·8; α-amino N/total N, 53. $C_{15}H_{22}O_4N_2$, H_2O requires C, 57·7; H, 7·7; α-amino N/total N, 50·0%.) The p*K* was 7·46.

(*b*) *O*-Acetyl-*N*-benzyloxycarbonyltyrosine (Blau & Waley, 1954) was condensed with leucine methyl ester to give *O-acetyl-N-benzyloxycarbonyltyrosylleucine methyl ester* (76%), which, after recrystallization from *iso*propyl ether:CCl_4, had m.p. 143°. (Found: C, 64·6; H, 6·6; N, 6·2; OMe, 6·5. $C_{26}H_{32}O_7N_2$ requires C, 64·5; H, 6·6; N, 5·8; OMe, 6·4%.) Hydrogenation in 80% (v/v) aqueous acetic acid containing 1·5 mol. prop. of HCl using 10% Pd-charcoal as catalyst gave *O-acetyltyrosylleucine methyl ester hydrochloride*, which was recrystallized from ethyl acetate: ether, m.p. 174–175° (decomp.). (Found: C, 55·6; H, 6·7; Cl, 9·7; OMe, 8·1. $C_{18}H_{28}O_5N_2$, HCl requires C, 55·9; H, 7·0; Cl, 9·2; OMe, 8·0%.) The methyl ester hydrochloride (0·66 g.) was dissolved in 0·74 N-$KHCO_3$ (5 ml.) and the free base isolated with ethyl acetate. After shaking for 30 min. with 0·1015 N-$Ba(OH)_2$ (67·4 ml.) the solution was neutralized with H_2SO_4, filtered and evaporated. Recrystallization from 80% ethanol gave tyrosylleucine hydrate, m.p. 278° (decomp.); $[\alpha]_D^{21}$ +6·9±0·1° in 0·5 N-HCl (*c*, 9·3). (Found: C, 57·4; H, 7·8; α-amino N/total N, 51. Calc. for $C_{15}H_{22}O_4N_2$, H_2O: C, 57·7; H, 7·7; α-amino N/total N, 50·0%.) The p*K* was 7·44.

ε-Benzyloxycarbonyllysine ethyl ester hydrochloride. ε-Benzyloxycarbonyl-α-carboxylysine anhydride (Bergmann *et al.* 1935) (12·2 g.) in ethanolic HCl (85 ml. of N) was heated to the b.p. and then allowed to cool. After distillation of the solvent, the *hydrochloride monohydrate* was recrystallized from ethyl acetate; yield 8·2 g., 60%, m.p. 115·5–116·5°; $[\alpha]_D^{17}$ +12·2±0·2° in 0·1 N-HCl (*c*, 2·1). (Found: N, 7·45; Cl, 9·8. $C_{16}H_{24}O_4N_2$, HCl, H_2O requires N, 7·7; Cl, 9·8%.)

Dibenzyloxycarbonyllysyl-ε-benzyloxycarbonyllysine ethyl ester. Dibenzyloxycarbonyllysine was condensed with the ε-benzyloxycarbonyllysine ethyl ester. The *product* was collected, and recrystallized from ethyl acetate: ether; yield 71%; m.p. 117–117·5°. $[\alpha]_D^{18}$ −5·6±0·2° in acetone (*c*, 2·46). (Found: C, 64·8; H, 6·9; N, 8·0. $C_{38}H_{48}O_9N_4$ requires C, 64·8; H, 6·8; N, 7·95%.)

Lysyllysine ethyl ester. Dibenzyloxycarbonyllysyl-ε-benzyloxycarbonyllysine ethyl ester was hydrogenated in ethanolic HCl, using Pd black as catalyst. The product could not be crystallized, but was chromatographically homogeneous.

Enzymic reactions

The enzymic reactions, carried out at 25·8°, were followed by transferring samples (approx. 10 μl.) at various times to the starting line of a chromatogram; a drop of 2 N acetic acid had previously been applied to this position. The chromatographic solvent was *n*-butanol:acetic acid:pyridine:water (30:6:20:24, v/v); Whatman no. 1 paper was customarily employed. The R_F values of the compounds studied are given in Table 1. Markers were used in 1% solution in 50% (v/v) aqueous *iso*propanol, and the chromatograms were developed by spraying with a solution of ninhydrin (0·4%) and cobalt chloride (0·2%) in *iso*propanol, followed by heating at 110° for 10 min. (Wiggins & Williams, 1952). Since both the α- and ε-amino groups of lysine react with ninhydrin, especially small amounts of lysine peptides are detectable, varying from 0·1 μg. in the case of dilysine to 0·9 μg. in the case of Lys.Tyr.Leu. Peptides containing tyrosine were revealed by spraying chromatograms with either Pauly reagent (Block, Le Strange & Zweig, 1952) or

Table 1. *R_F values of reference compounds*

The compounds were chromatographed on Whatman no. 1 paper using *n*-butanol:acetic acid:pyridine:water (30:6:20:24, v/v).

Substance	R_F
Lysine	0·24
Dilysine	0·17
Trilysine	0·12
Tetralysine	0·09
Pentalysine	0·06
Lysine amide	0·29
Lysyltyrosine amide	0·51
Lysyllysyltyrosine amide	0·36
Tyrosine	0·56
Leucine	0·66
Tyrosyllysine	0·42
Tyrosylleucine	0·83
Lysyltyrosyllysine	0·33
Lysyltyrosylleucine	0·64
Lysyllysine ethyl ester	0·38

Folin's reagent (Cornforth, Cornforth, Dalgliesh & Neuberger, 1951); with the latter reagent, the smallest quantity of peptide which could be detected was the amount which contained about 0·3 μg. of tyrosine.

Experiments with dilysine, lysine amide, Lys.Lys.Tyr amide, Lys.Tyr.Lys and Lys.Tyr.Leu, under the same conditions of concentration, temperature and pH as were used in the enzymic reactions, showed that no decomposition could be detected within 24 hr. The case of dilysine ethyl ester is discussed in detail on p. 536.

RESULTS AND DISCUSSION

The following abbreviations are used: Lys for lysine and lysyl residues, Tyr for tyrosine and tyrosyl residues, Leu for leucyl residues, Gly for glycyl residues and Am and Et for amide and ethyl ester groups respectively.

Dilysine. Although it has been reported that trypsin does not attack dilysine (Waley & Watson, 1953*b*), it has now been found that at relatively high concentrations of the enzyme reaction does take place; the course of the reaction is shown in Fig. 1.

As far as one can tell by visual inspection, comparable amounts of lysine and trilysine are formed initially and only in the later stages of the reaction, when trilysine will have decomposed somewhat (equation 2), is the amount of lysine appreciably greater than the amount of trilysine. The trilysine is formed by the transpeptidation reaction 1.

$$2\ \text{Lys.Lys} \rightarrow \text{Lys} + \text{Lys.Lys.Lys} \qquad (1)$$

$$\text{Lys.Lys.Lys} \rightarrow \text{Lys} + \text{Lys.Lys} \qquad (2)$$

Equation 1 gives equimolecular amounts of lysine and trilysine. Now if any direct hydrolysis of dilysine (equation 3) occurred, the amount of lysine would always be greater than the amount of trilysine; this does not appear to be the case, so that direct hydrolysis does not take place.

$$\text{Lys.Lys} \rightarrow 2\ \text{Lys} \qquad (3)$$

Transpeptidations leading to higher peptides of lysine also occur to a small extent and at higher substrate concentrations (10%) greater amounts of tetralysine are formed. Experiments carried out at pH values from 6 to 10 showed a broad optimum about pH 8. The intermediate trilysine is relatively stable and thus transpeptidation is readily detected

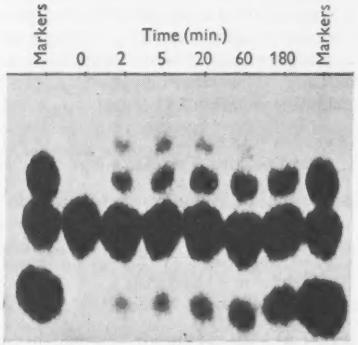


Fig. 1. Chromatogram showing the action of trypsin on dilysine dihydrochloride. A 4% (w/v) solution of trypsin in 0·001 N-HCl (0·25 ml.), which had been dialysed against 0·001 N-HCl for 1 hr., was added to dilysine dihydrochloride (19·6 mg.) in 0·27 N-$KHCO_3$ (0·75 ml.). If the $MgSO_4$ is not removed from the trypsin solution by dialysis, the lysine spot on paper chromatograms is split into two spots. The $MgSO_4$ does not interfere at low concentrations.

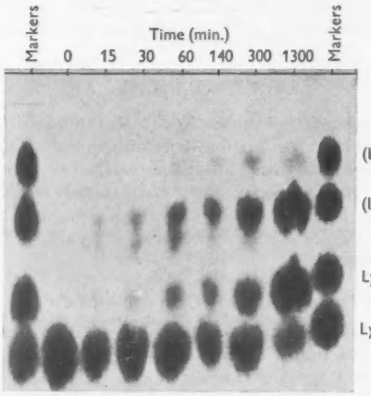


Fig. 2. Chromatogram showing the action of trypsin on lysine amide dihydrochloride. A 4% solution of trypsin in 0·001 N-HCl (0·06 ml.), which had been dialysed against 0·001 N-HCl for 1 hr., was added to lysine amide dihydrochloride (5·0 mg.) in phosphate buffer, pH 7·1 (Clark & Lubs, 1916) (0·14 ml.) containing 0·2 N-NaOH (0·055 ml.).

in this case, but the pattern of reactions 1 and 2, which together furnish a mode of hydrolysis, may be quite common and if the second step in such a sequence is considerably faster than the first the intermediate formed by transpeptidation will never be present in sufficient quantity to be detected. Thus transpeptidation may occur even when it is not detected. Since many important biological compounds are built up and broken down by cyclic processes, it is interesting that the decomposition of dilysine should be a cyclic process of a particularly simple kind; the second molecule of dilysine may be considered to act as a cosubstrate (Behrens & Bergmann, 1939).

Lysine amide. The course of the reaction is shown in Fig. 2.

The main products are lysine and dilysine (which are formed in comparable amounts). Trilysine is also formed and an intermediate whose R_F is slightly higher than dilysine; this is believed to be dilysine amide. Thus, the reactions taking place may be

$$2\ \text{Lys.Am} \rightarrow \text{Lys.Lys.Am} + \text{NH}_3 \quad (4)$$

$$\text{Lys.Lys.Am} \rightarrow \text{Lys.Lys} + \text{NH}_3 \quad (5)$$

$$\text{Lys.Lys.Am} \rightarrow \text{Lys} + \text{Lys.Am} \quad (6)$$

$$\text{Lys.Am} + \text{Lys.Lys} \rightarrow \text{Lys.Lys.Lys} + \text{NH}_3 \quad (7)$$

Alternatively, the trilysine may be formed by transpeptidation from two molecules of dilysine, instead of from lysine amide and dilysine. There are several other reactions which may be postulated, but as there is no definite evidence of their occurring they will not be mentioned. The chief point is that dilysine, which is a major product, must arise by transpeptidation.

The pH optimum was about 7; at higher pH values, relatively more trilysine and dilysine were formed; at lower pH values the reaction was very slow.

Lysyllysyltyrosine amide. The course of the reaction is shown in Fig. 3. As the substrate decomposed, dilysine, tetralysine and tyrosine amide accumulated. Then the tetralysine disappeared. A separate chromatogram was sprayed with Folin's reagent: this revealed only the Lys.Lys.Tyr amide and tyrosine amide spots. The spot between di- and tetra-lysine (Fig. 3) did not contain any tyrosine residues and is assumed to be trilysine. No lysine spot is observable; a small amount of Lys.Tyr amide was also formed; it is not detectable in the photograph (Fig. 3).

One of the main reactions is apparently

$$\text{Lys.Lys.Tyr.Am} \rightarrow \text{Lys.Lys} + \text{Tyr.Am} \quad (8)$$

The tetralysine may arise as follows:

$$\text{Lys.Lys.Tyr.Am} + \text{Lys.Lys} \rightarrow \text{Lys.Lys.Lys.Lys} + \text{Tyr.Am} \quad (9)$$

The trilysine cannot arise from decomposition of the tetralysine since no lysine is formed; it must therefore arise by transpeptidation reactions, such as

Lys.Lys.Tyr.Am + Lys.Lys
→ Lys.Lys.Lys + Lys.Tyr.Am (10)

or

Lys.Lys.Tyr.Am + Lys.Tyr.Am
→ Lys.Lys.Lys.Tyr.Am + Tyr.Am (11)

Lys.Lys.Lys.Tyr.Am
→ Lys.Lys.Lys + Tyr.Am (12)

The posulated intermediate, Lys.Lys.Lys.Tyr amide, would be expected to be detectable at a concentration of approximately 0·01% (by spraying with Folin reagent), but no tyrosine-containing peptide was observed of R_F lower than that of the starting material. If the rate of decomposition of Lys.Lys.Lys.Tyr amide were much greater than its rate of formation, however, its concentration would always be very low.

The action of trypsin on a mixture of Lys.Lys.Tyr amide (2%) and tyrosine amide (5%) showed several differences. Tetralysine was no longer formed, more Lys.Tyr amide appeared, and the starting material disappeared more slowly. The Lys.Tyr amide may arise from

Lys.Lys.Tyr.Am + Tyr.Am → 2 Lys.Tyr.Am (13)

Trilysine presumably arises from reaction 13, followed by reactions 11 and 12. This sequence is suggested to account for more trilysine being formed when tyrosine amide is present; the other reaction 10, which gives rise to trilysine, depends on the participation not of tyrosine amide but of dilysine.

There are two possible explanations of the fact that the addition of tyrosine amide causes the lysyllysyltyrosine amide to decompose more slowly. The tyrosine amide may combine with the trypsin, but since trypsin is only known to combine with amino acid residues bearing a basic side chain this appears unlikely. The other explanation is that a transpeptidation reaction 14 occurs:

Lys.Lys.Tyr.Am + Tyr.Am
⇄ Lys.Lys.Tyr.Am + Tyr.Am (14)

This transpeptidation is analogous to reaction 15 which Johnston, Mycek & Fruton (1950) discovered, using isotopically labelled glycine amide (Gly*.Am).

Benzoyl.Tyr.Gly.Am + Gly*.Am
⇄ Benzoyl.Tyr.Gly*.Am + Gly.Am (15)

That the transpeptidation reaction 14 would decrease the rate of disappearance of Lys.Lys.Tyr amide can be shown as follows. If we restrict ourselves to the effect of reaction 14 on the rate of the direct hydrolysis (reaction 8), then if the Lys.Lys.Tyr amide is written as A, the tyrosine amide as B, the dilysine as C, and the trypsin as E, the reaction scheme may be written

$$A + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} (AE)$$

$$(AE) \xrightarrow{k_3} B + C + E$$

$$(AE) + B \xrightarrow{k_4} B + A + E.$$

We will assume that the stationary-state approximation applies to the enzyme–substrate complex, and that the concentration of tyrosine amide (which was present in excess in our experiments) remains constant. Then it may readily be shown that the half-life ($t_{\frac{1}{2}}$) for the disappearance of Lys.Lys.Tyr amide is given by

$$t_{\frac{1}{2}} = \{(K_m + k_4 B_o/k_1) \log 2\}/k_3 e + A_o/2k_3 e,$$

where e is the total concentration of trypsin, A_o and B_o are the initial concentrations of Lys.Lys.Tyr amide and tyrosine amide, and $K_m = (k_2 + k_3)/k_1$. Thus the half-life increases with the initial concentration of tyrosine amide.

The following transpeptidation between Lys.Lys.Tyr amide and phenylalanine amide, which is analogous to reaction 14, has also been observed.

Lys.Lys.Tyr.Am + Phe.Am
→ Lys.Lys.Phe.Am + Tyr.Am (16)

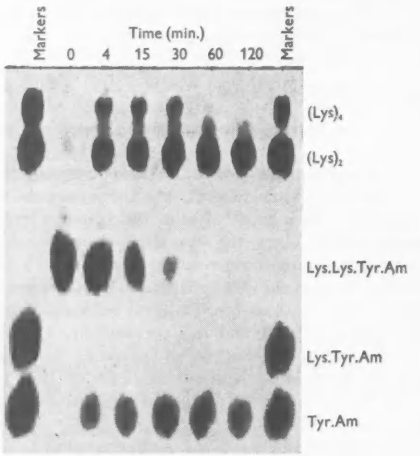


Fig. 3. Chromatogram showing the action of trypsin on lysyllysyltyrosine amide trihydrochloride. Trypsin (0·22 mg.) in water (0·22 ml.) was added to Lys.Lys.Tyr amide trihydrochloride (8·8 mg.) in 0·4N-$KHCO_3$ (0·22 ml.).

Pentalysine and tyrosine amide. Without added tyrosine amide, trypsin breaks pentalysine down to dilysine and trilysine in equimolecular amounts. When tyrosine amide was added, initially more dilysine than trilysine was formed; in addition, two new compounds (R_F values 0·36 and 0·29) appeared, also in unequal amounts. Since lysyllysyltyrosine amide has R_F 0·36, this is taken to be one of the new compounds; the other, formed in greater amount, is probably lysyllysyllysyltyrosine amide. These two compounds decomposed at later stages of the reaction, and the amounts of dilysine and trilysine became equal.

Thus, we postulate the transpeptidations 17 and 18 and apparently reaction 18 is faster.

$$\text{Lys.Lys.Lys.Lys.Lys} + \text{Tyr.Am} \rightarrow \text{Lys.Lys.Tyr.Am} + \text{Lys.Lys.Lys} \quad (17)$$

$$\text{Lys.Lys.Lys.Lys.Lys} + \text{Tyr.Am} \rightarrow \text{Lys.Lys.Lys.Tyr.Am} + \text{Lys.Lys} \quad (18)$$

Similar results were obtained by the action of trypsin on polylysine in the presence of tyrosine amide: di-, tri- and tetra-lysine were formed as usual (Waley & Watson, 1953*b*) and in addition lysyltyrosine amide and lysyllysyltyrosine amide appeared. Higher members of the series $(\text{lysyl})_n$-tyrosine amide were probably also present, as several tyrosine-containing compounds with relatively low R_F values were detected by spraying with Pauly reagent.

Lysyltyrosyllysine. The course of the reaction is shown in Fig. 4.

After 1300 min. all the lysyltyrosyllysine had reacted and the products were dilysine, lysine and tyrosyllysine; the dilysine must arise by a transpeptidation reaction. In the early stages of the reaction (up to 40 min.) a product was formed with R_F intermediate between lysine and dilysine (Fig. 4); this product, which was shown to contain tyrosine by spraying with Pauly reagent, is, together with tyrosyllysine, clearly a primary product. This substance is believed to be lysyllysyltyrosyllysine, arising by reaction 19 and decomposing to give dilysine by reaction 20.

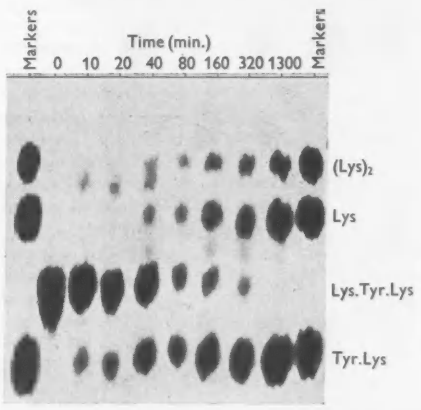


Fig. 4. Chromatogram showing the action of trypsin on lysyltyrosyllysine dihydrochloride. Trypsin (0·88 mg.) in water (0·22 ml.) was added to lysyltyrosyllysine dihydrochloride (8·6 mg.) in 0·2 N-$KHCO_3$ (0·22 ml.).

$$2\ \text{Lys.Tyr.Lys} \rightarrow \text{Tyr.Lys} + \text{Lys.Lys.Tyr.Lys} \quad (19)$$

$$\text{Lys.Lys.Tyr.Lys} \rightarrow \text{Lys.Lys} + \text{Tyr.Lys} \quad (20)$$

The lysine formed might arise by hydrolysis of the starting material:

$$\text{Lys.Tyr.Lys} \rightarrow \text{Lys} + \text{Tyr.Lys} \quad (21)$$

However, Fig. 4 shows that lysine only appears after 40 min., when the dilysine appears. Since the dilysine arises by break-down of the tetrapeptide lysyllysyltyrosyllysine, it seems likely that the lysine is similarly formed (reaction 22).

$$\text{Lys.Lys.Tyr.Lys} \rightarrow \text{Lys} + \text{Lys.Tyr.Lys} \quad (22)$$

The sequence of reactions 19 and 22 constitute a cyclic process, analogous to reactions 1 and 2.

No new products appear to be formed by the addition of leucine, or arginine, to the reaction mixture; but in the presence of tyrosylleucine, some lysyltyrosylleucine is formed, and in the presence of tyrosine amide some lysyltyrosine amide.

Lysyltyrosylleucine. This substrate does not react with trypsin as fast as lysyltyrosyllysine does; dilysine, lysine and tyrosylleucine are formed. In this case no tetrapeptide intermediate could be detected, but it seems likely that dilysine is formed by reactions 23 and 24, analogous to reactions 19 and 20.

$$2\ \text{Lys.Tyr.Leu} \rightarrow \text{Tyr.Leu} + \text{Lys.Lys.Tyr.Leu} \quad (23)$$

$$\text{Lys.Lys.Tyr.Leu} \rightarrow \text{Lys.Lys} + \text{Tyr.Leu} \quad (24)$$

If the second step takes place much more rapidly than the first, the concentration of lysyllysyltyrosylleucine will always be low.

Dilysine ethyl ester. The course of the reaction is shown in Fig. 5.

The first product of the reaction is dilysine; subsequently tetralysine is formed. Dilysine ethyl ester is very readily attacked by trypsin and at the low trypsin concentrations used the tetralysine formed does not decompose at all rapidly. Traces of a substance *X* (R_F 0·29) also appear; its structure has not yet been determined, but it may well be the diketopiperazine of lysine.

There are two ways in which the tetralysine might arise (reactions 25 and 26 or 27 and 28):

$$\text{Lys.Lys.Et} \rightarrow \text{Lys.Lys} + \text{EtOH} \quad (25)$$

$$\text{Lys.Lys} + \text{Lys.Lys.Et} \rightarrow \text{Lys.Lys.Lys.Lys} + \text{EtOH} \quad (26)$$

$$2\ \text{Lys.Lys.Et} \rightarrow \text{Lys.Lys.Lys.Lys.Et} + \text{EtOH} \quad (27)$$

$$\text{Lys.Lys.Lys.Lys.Et} \rightarrow \text{Lys.Lys.Lys.Lys} + \text{EtOH} \quad (28)$$

No evidence for the presence of tetralysine ethyl ester has been found. It might be unstable and only present in low concentrations, or its R_F might be the same as that of dilysine so that its presence would remain undetected. Again, dilysine might arise by direct hydrolysis of dilysine ethyl ester or by reaction 29.

$$\text{Lys.Lys.Lys.Lys.Et} \rightarrow \text{Lys.Lys} + \text{Lys.Lys.Et} \quad (29)$$

Variation of pH altered the nature of the products formed, but the rate did not vary greatly between pH values of 6 and 11. At pH 6 the sole product was dilysine. At pH 10 more tetralysine was formed than dilysine and some products with lower R_F values appeared; these are believed to be hexalysine and octalysine; similar products were formed at pH 11.

Some experiments were also carried out without any enzyme. At pH 8·3, the main product was *X*, and the only other product was dilysine; the dilysine ethyl ester was decomposed about 50 times more slowly in the absence of the enzyme than in the experiment at pH 8·3 described above. At higher pH values, the reactions in the absence of the enzyme were more rapid than those at pH 8·3, *X* and dilysine were again the main products (but relatively more dilysine was formed) and traces of tetra- and hexa-lysine were discernible.

The course of an experiment in which tyrosylleucine was added to dilysine ethyl ester is shown in Fig. 6.

The main products were dilysine, tetralysine and a further substance (R_F 0·53) which contained tyrosine; this substance was not stable, and was nearly all decomposed after 1000 min.; it is believed to be lysyllysyltyrosylleucine, formed by reaction 30.

$$\text{Lys.Lys.Et} + \text{Tyr.Leu} \rightarrow \text{Lys.Lys.Tyr.Leu} + \text{EtOH} \quad (30)$$

When tyrosyltyrosine amide replaced tyrosylleucine in the experiment described above, the products were dilysine, tetralysine and a substance

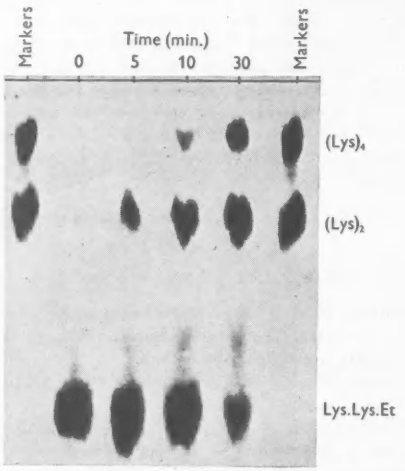


Fig. 5. Chromatogram showing the action of trypsin on dilysine ethyl ester trihydrochloride. Trypsin (12·5 μg.) in water (0·05 ml.) was added to dilysine ethyl ester trihydrochloride (9·4 mg.) in 0·2 N-$KHCO_3$ (0·45 ml.). The pH of the reaction mixture was 8·3.

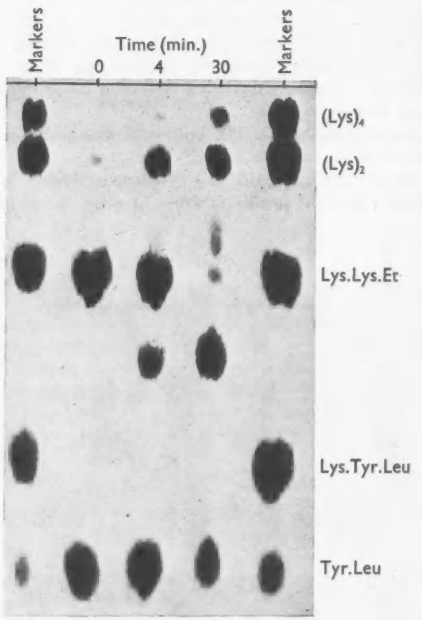


Fig. 6. Chromatogram showing the action of trypsin on dilysine ethyl ester trihydrochloride and tyrosylleucine. Trypsin (20 μg.) in water (0·08 ml.) was added to dilysine ethyl ester trihydrochloride (15·3 mg.) and tyrosylleucine (20·6 mg.) in 0·2 N-$KHCO_3$ (0·72 ml.).

(R_F 0·57) which is probably lysyllysyltyrosyltyrosine amide:

Lys.Lys.Et + Tyr.Tyr.Am → Lys.Lys.Tyr.Tyr.Am + EtOH (31)

Similarly, lysyllysine ethyl ester reacted with tyrosine amide to give lysyllysyltyrosine amide (in this case a synthetic specimen was available for comparison):

Lys.Lys.Et + Tyr.Am → Lys.Lys.Tyr.Am + EtOH (32)

When the reaction was carried out with excess of tyrosine amide (0·049M dilysine ester, 0·28M tyrosine amide), the dilysine ester reacted rapidly and completely with tyrosine amide; no dilysine or tetralysine were formed. The lysyllysyltyrosine amide so formed then decomposed slowly as described on p. 533. In the absence of enzyme, dilysine ester decomposed slowly in the presence of tyrosine amide at pH 8·3 to give some lysyllysyltyrosine amide; the main product was *X*.

Dilysine ethyl ester and lysyllysyltyrosine amide have the same R_F, but can easily be distinguished by spraying with Pauly reagent, which is specific for tyrosine residues. Phenylalanine amide reacted in the same way as tyrosine amide; in this case the formation of lysyllysylphenylalanine amide, which also had the same R_F as dilysine ester, was demonstrated chromatographically by elution of the material and partial acid hydrolysis: dilysine, lysine and phenylalanine were present.

Frequency of occurrence of transpeptidation. Our experiments with trypsin show that transpeptidation occurs frequently. The substrates used, arranged in order of decreasing reactivity, are: dilysine ethyl ester, pentalysine, lysyllysyltyrosine amide, lysyltyrosyllysine, lysine amide, lysyltyrosylleucine and dilysine. A wide range of reactivity is covered by these compounds; dilysine ethyl ester is about 10^4 times as reactive as dilysine, so transpeptidation is not restricted to the less reactive substrates. Furthermore, both the least and the most reactive substrates can, under certain conditions, undergo transfer reactions without any concomitant hydrolysis.

Role of transpeptidations in vivo. It seems reasonable to suppose that transpeptidations may play a part in the metabolism of proteins. There are two effects which transpeptidation may have: (*a*) to lengthen one peptide chain at the expense of another, (*b*) to produce novel sequences of amino acids. One of the chief reasons for considering that the first effect may mediate in protein synthesis is that the peptide chain may be lengthened with relatively little change in free energy. The second effect may accelerate the enzymic hydrolysis of proteins by substituting a more readily hydrolysed peptide bond for a resistant one.

SUMMARY

1. Some of the methods available for the synthesis of peptides are discussed. The azide method and the anhydride method of Wieland & Sehring (1950) have been used to prepare lysyltyrosyllysine and lysyltyrosylleucine; several other peptides of lysine, tyrosine and leucine have also been synthesized.

2. The action of trypsin on dilysine, lysine amide, lysyllysyltyrosine amide, lysyltyrosyllysine and lysyltyrosylleucine has been examined chromatographically. In all these cases, transpeptidations take place. Trypsin also catalyses transpeptidation between pentalysine and tyrosine amide.

3. Dilysine ethyl ester has been found to be a very reactive substrate for trypsin. Both hydrolysis and condensation to give polymers took place, as did condensation with tyrosine amide, phenylalanine amide, tyrosyltyrosine amide and tyrosylleucine.

4. In some cases hydrolysis of a peptide apparently takes place by way of an intermediate formed by transpeptidation. Some of the results are interpreted in terms of cyclic reactions.

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Chymotrypsin-Catalysed Transpeptidations

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(*Received* 8 *December* 1953)

The transferase activity of the proteolytic enzyme trypsin has recently been investigated (see preceding paper, Waley & Watson, 1954); this work has now been extended to chymotrypsin. The substrates used were tyrosine amide, phenylalanine amide, tyrosyltyrosine amide, phenylalanylphenylalanine amide, tyrosylphenylalanine amide, phenylalanyltyrosine amide, and tyrosyltyrosine. Most of these substrates were originally investigated by Fruton & Bergmann (1942), who implicitly assumed that hydrolysis was the sole chymotrypsin-catalysed reaction.

Hydrolysis is indeed the final result, but we have shown chromatographically that transpeptidation also occurs. Moreover, the initial reaction may be entirely transpeptidation, with subsequent hydrolysis of the intermediates so formed.

EXPERIMENTAL

General. Crystalline chymotrypsin (not more than 50% $MgSO_4$, $7H_2O$) and crystalline pepsin were obtained from Armour Laboratories Ltd., Lindsey Street, London, E.C. 1. Papain (Hopkin & Williams Ltd.) was purified as described by Grassmann (1935). Ficin (L. Light and Co. Ltd.) was used as supplied. Microanalyses were carried out by Mr G. Ingram of these laboratories. Melting points are uncorrected. The error of the optical rotations is the standard deviation (Dean & Dixon, 1951). The amino acids used all had the L configuration.

Materials

Tyrosine amide. Tyrosine methyl ester (Fischer & Schrauth, 1907) was allowed to stand for 48 hr. in methanol saturated with NH_3. After evaporation of the solution, the residue was recrystallized from ethanol (yield, 82%). After two further recrystallizations the tyrosine amide had m.p. 153–154°; $[\alpha]_D^{20}$ +19·2±0·1° in water (*c*, 4·1). Koenigs & Mylo (1908) give m.p. 153–154°; $[\alpha]_D^{20}$ +19·5°. (Found: C, 60·3; H, 7·0; N, 15·6; equiv. wt. 180. Calc. for $C_9H_{12}O_2N_2$: C, 60·0; H, 6·7; N, 15·5%; equiv. wt. 180.)

N-Benzyloxycarbonyltyrosine methyl ester. The method of Bergmann & Zervas (1932) was applied to tyrosine methyl ester. After recrystallization from di*iso*propyl ether, *N-benzyloxycarbonyltyrosine methyl ester* had m.p. 94–95°; $[\alpha]_D^{20}$ −6·1±0·1° in ethanol (*c*, 2·0). (Found: C, 66·0; H, 5·9. $C_{18}H_{19}O_5N$ requires C, 65·6; H, 5·8%.)

O-Acetyl-N-benzyloxycarbonyltyrosine. *N*-Benzyloxycarbonyltyrosine methyl ester was hydrolysed and acetylated by the method of Bergmann, Zervas, Salzmann & Schleich (1934). The crude product, isolated by extraction with ethyl acetate, was recrystallized from di*iso*propyl ether. *O*-Acetyl-*N*-benzyloxycarbonyltyrosine had m.p. 112–113°; $[\alpha]_D^{20}$ +3·2±0·2° in ethanol (*c*, 2·3). Bergmann, Zervas, Salzmann & Schleich (1934) give m.p. 120–121° (not sharp); the m.p. is variable unless the specimen is carefully dried. (Found: C, 63·8; H, 5·2; N, 4·1. Calc. for $C_{19}H_{19}O_6N$: C, 63·9; H, 5·3; N, 3·9%.)

O-Acetyl-N-benzyloxycarbonyltyrosyltyrosine amide. The peptide synthesis of Wieland & Sehring (1950) was used. Benzoyl chloride (4·6 ml.) was added to *O*-acetyl-*N*-benzyloxycarbonyltyrosine (14·3 g.) and *N*-ethylpiperidine (5·4 ml.) in tetrahydrofuran (50 ml.) at 0°. After 10 min. the mixture was added to tyrosine amide (7·2 g.) suspended in ethyl acetate (50 ml.) and 1·3N-$KHCO_3$ (50 ml.). The mixture was stirred for 1 hr., and the product collected, washed with water and ether and recrystallized from methanol. *O-Acetyl-N-benzyloxycarbonyltyrosyltyrosine amide* (10·7 g., 51%) had m.p. 209–211°; $[\alpha]_D^{20}$ −14·9±0·2° in dioxan (*c*, 2·4). (Found: C, 64·8; H, 5·7; N, 8·1. $C_{28}H_{29}O_7N_3$ requires C, 64·7; H, 5·6; N, 8·1%.)

Tyrosyltyrosine amide. *O*-Acetyl-*N*-benzyloxycarbonyltyrosyltyrosine amide was suspended in 0·2N methanolic HCl and hydrogenated using 10% Pd-charcoal. The use of acid in the reduction medium removes the acetyl as well as the benzyloxycarbonyl group (Barkdoll & Ross, 1944). The filtered solution was evaporated, the residue dissolved in the minimum amount of water and the pH brought to 7·0. The product crystallized slowly. *Tyrosyltyrosine amide* had m.p. 193–194°; $[\alpha]_D^{20}$ +4·7±0·6° in methanol (*c*, 1·7).

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(Found: C, 63·4; H, 6·2; N, 11·9; equiv. wt. 348. $C_{18}H_{21}O_4N_3$ requires C, 63·0; H, 6·2; N, 12·2%; equiv. wt. 343.)

Phenylalanine amide. Phenylalanine methyl ester (Flowers & Reith, 1953) was converted into the amide as described for tyrosine amide. After recrystallization from ethyl acetate, phenylalanine amide had m.p. 93–94°; $[\alpha]_D^{20} + 20{\cdot}7 \pm 0{\cdot}2°$ in water (*c*, 1·75).

N - *Benzyloxycarbonylphenylalanylphenylalanine amide.* *N*-Benzyloxycarbonylphenylalanine (Bergmann, Zervas, Rinke & Schleich, 1934) was condensed with phenylalanine amide by the method described above. *N*-Benzyloxycarbonylphenylalanylphenylalanine amide had m.p. 228–229°. Fruton & Bergmann (1942) give m.p. 230°. (Found: C, 70·3; H, 6·2; N, 9·5. Calc. for $C_{28}H_{27}O_4N_3$: C, 70·1; H, 6·1; N, 9·4%.)

O-*Acetyl*-N-*benzyloxycarbonyltyrosylphenylalanine amide.* *O*-Acetyl-*N*-benzyloxycarbonyltyrosine was condensed with phenylalanine amide as described above, and the product recrystallized from dioxan. O-*Acetyl*-N-*benzyloxycarbonyltyrosylphenylalanine amide* had m.p. 225–226°. (Found: C, 66·8; H, 5·8; N, 8·4. $C_{28}H_{29}O_6N_3$ requires C, 66·7; H, 5·8; N, 8·3%.)

Tyrosylphenylalanine amide. *O*-Acetyl-*N*-benzyloxycarbonyltyrosylphenylalanine amide was suspended in 0·2N methanolic HCl and hydrogenated using 10% Pd-charcoal. Neither tyrosylphenylalanine amide hydrochloride, nor the free base, could be purified but they were chromatographically homogeneous, and gave the expected products on acid and enzymic hydrolysis.

N-*Benzyloxycarbonylphenylalanyltyrosine amide.* *N*-Benzyloxycarbonylphenylalanine was condensed with tyrosine amide and the product recrystallized from methanol. *N*-Benzyloxycarbonylphenylalanyltyrosine amide had m.p. 220–221°. Fruton & Bergmann (1942) give m.p. 221°. (Found: C, 67·6; H, 6·0; N, 9·3. Calc. for $C_{28}H_{27}O_5N_3$: C, 67·7; H, 5·9; N, 9·1%.)

Enzymic reactions

In the enzymic reactions, carried out at 25·8°, the substrate concentration was normally 0·05M and the enzyme concentration was 2%. The reactions were followed by transferring 10 µl. samples at various times to positions on the starting line of a paper chromatogram. Whatman no. 1 paper which had been previously treated with 2N acetic acid, was used. The chromatographic solvents were (by vol.): *n*-butanol:acetic acid:pyridine:water (30:6:20:24; Waley & Watson, 1953); pyridine:*iso*amyl alcohol:water (7:7:6; Edman, 1946) and pyridine:*iso*amyl alcohol:water (37:43:20). The R_F values of the compounds used in this work are given in Table 1. Tyrosine-containing compounds were revealed by spraying with the Pauly reagent (Block, Le Strange & Zweig, 1952). The two-dimensional chromatograms were run on Whatman no. 3MM paper, developed first with *n*-butanol saturated with water and then with pyridine:*iso*amyl alcohol:water (Edman, 1946). It was found that the paper could be sprayed first with Pauly reagent and the spots outlined. After drying, the other side was sprayed with a solution of ninhydrin (0·4%) and cobalt chloride (0·2%) in *iso*propanol (Wiggins & Williams, 1952).

RESULTS

The following abbreviations are used: Tyr for tyrosine and tyrosyl residues, Phe for phenylalanine and phenylalanyl residues, and Am for amide groups.

Chymotrypsin

Tyrosyltyrosine amide. The course of the reaction is shown in Fig. 1.

The products are tyrosine, tyrosyltyrosine, and tyrosine amide; thus both the peptide and the amide bonds are split:

$$\text{Tyr.Tyr.Am} \rightarrow \text{Tyr} + \text{Tyr.Am} \quad (1)$$

$$\text{Tyr.Tyr.Am} \rightarrow \text{Tyr.Tyr} + \text{NH}_3 \quad (2)$$

Equimolecular amounts of tyrosine and tyrosine amide should therefore be formed. It appears, however, that less tyrosine is formed; it seems possible that a reaction of the following type may occur.

$$\text{Tyr.Tyr.Am} + \text{Tyr} \rightarrow \text{Tyr.Tyr} + \text{Tyr.Am} \quad (3)$$

The tyrosine amide formed in reactions 1 and 3 will react further as described below.

Table 1. *R_F values of reference compounds*

The compounds were chromatographed on Whatman no. 1 paper.

Substance	Solvent: Pyridine:*iso*amyl alcohol:water (7:7:6, v/v)	Pyridine:*iso*amyl alcohol:water (37:43:20, v/v)	*n*-Butanol:acetic acid:pyridine:water (30:6:20:24, v/v)	*n*-Butanol saturated with water
Tyr	0·47	0·30	0·55	0·21
Tyr.Am	0·62	0·53	0·64	0·27
Tyr.Tyr	0·64	0·47	0·76	0·20
Tyr.Tyr.Am	0·81	0·75	0·80	—
Tyr.Tyr.Tyr	0·71	—	0·86	—
Phe	0·51	—	0·65	0·33
Phe.Am	0·68	—	0·72	0·40
Phe.Phe	0·64	—	0·84	0·52
Phe.Phe.Am	0·86	—	0·86	0·69
Tyr.Phe	0·65	—	0·81	0·47
Tyr.Phe.Am	0·81	—	0·89	0·56
Phe.Tyr	0·56	—	0·82	0·37
Phe.Tyr.Am	0·82	—	0·85	0·62

Phenylalanylphenylalanine amide (Fruton & Bergmann, 1942) reacted similarly.

Tyrosine amide. The course of the reaction is shown in Fig. 2.

The products are tyrosine and tyrosyltyrosine. The tyrosine may arise by direct hydrolysis.

$$\text{Tyr.Am} \rightarrow \text{Tyr} + \text{NH}_3 \qquad (4)$$

The tyrosyltyrosine is probably formed by the hydrolysis of tyrosyltyrosine amide (reaction 2) which may arise by transpeptidation from tyrosine amide.

$$2\ \text{Tyr.Am} \rightarrow \text{Tyr.Tyr.Am} + \text{NH}_3 \qquad (5)$$

It appears from Fig. 2 that initially more tyrosine than tyrosyltyrosine is formed, but that later the relative amounts are reversed. This could be caused by the occurrence of reaction 3. The tyrosyltyrosine amide postulated as an intermediate is so reactive in the presence of the enzyme, that its stationary concentration is very low.

Chymotrypsin shows the usual pH optimum of about pH 8 in this reaction.

The final product of the decomposition of tyrosine amide is tyrosine, since tyrosyltyrosine is slowly hydrolysed, as shown below.

Phenylalanine amide gave analogous results to those obtained with tyrosine amide, but reacted more slowly.

Tyrosyltyrosine (Bergmann, Zervas, Salzmann & Schleich, 1934). The main reaction here appears to be hydrolysis, which is comparatively slow:

$$\text{Tyr.Tyr} \rightarrow 2\ \text{Tyr} \qquad (6)$$

Using a high initial substrate concentration (6%), tyrosyltyrosyltyrosine is also formed.

$$2\ \text{Tyr.Tyr} \rightarrow \text{Tyr.Tyr.Tyr} + \text{Tyr} \qquad (7)$$

This was identified by comparing its R_F value with that of one of the products obtained by refluxing poly-(*O*-acetyl)tyrosine for 15 min. with constant b.p. hydrobromic acid (Abbott & Waley, unpublished work).

Tyrosylphenylalanine amide. The enzymic reaction was carried out in 30% (v/v) aqueous methanol. The main products were tyrosine and phenylalanine amide. Phenylalanine and tyrosylphenylalanine appeared later:

$$\text{Tyr.Phe.Am} \rightarrow \text{Tyr} + \text{Phe.Am} \qquad (8)$$

$$\text{Tyr.Phe.Am} \rightarrow \text{Tyr.Phe} + \text{NH}_3 \qquad (9)$$

Phenylalanyltyrosine amide (Fruton & Bergmann, 1942). This reaction was also carried out in 30% methanol. The main products were formed as follows:

$$\text{Phe.Tyr.Am} \rightarrow \text{Phe} + \text{Tyr.Am} \qquad (10)$$

$$\text{Phe.Tyr.Am} \rightarrow \text{Phe.Tyr} + \text{NH}_3 \qquad (11)$$

The tyrosine amide also gave the usual decomposition products (see above).

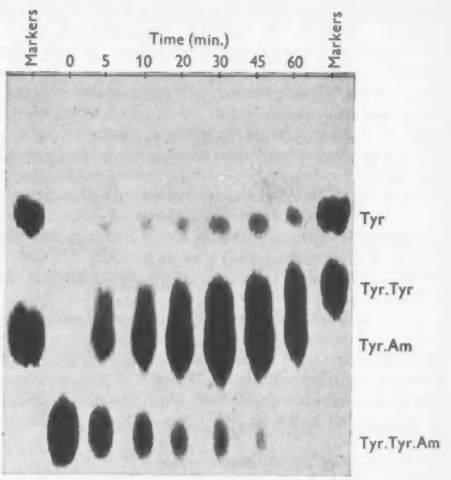

Fig. 1. Chromatogram showing the action of chymotrypsin on tyrosyltyrosine amide. Chymotrypsin (1 mg.) was added to tyrosyltyrosine amide hydrochloride (9·5 mg.) in phosphate buffer, pH 7·8 (Clark & Lubs, 1916) (0·225 ml.) and N-NaOH (0·025 ml.). This gives a supersaturated solution of tyrosyltyrosine amide. The chromatographic solvent was pyridine: *iso*amyl alcohol: water (37:43:20, v/v).

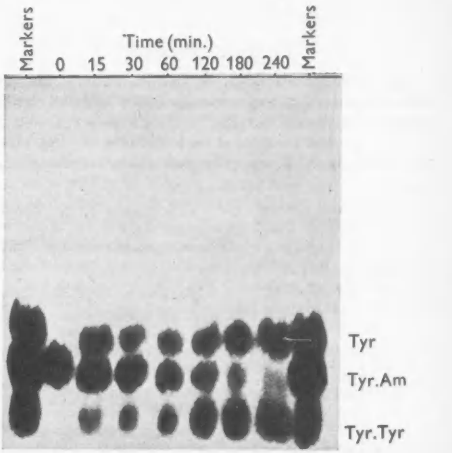

Fig. 2. Chromatogram showing the action of chymotrypsin on tyrosine amide. Chymotrypsin (20 mg.) was added to tyrosine amide (9·1 mg.) in phosphate buffer, pH 7·8 (Clark & Lubs, 1916) (1·0 ml.). The chromatographic solvent used was *n*-butanol: acetic acid: pyridine: water (30:6:20:24, v/v).

Tyrosine amide and phenylalanine amide. Equimolecular amounts of the two substrates were used. The products, identified by two-dimensional chromatography, were tyrosine, phenylalanine, tyrosyltyrosine, phenylalanylphenylalanine and phenylalanyltyrosine. The last might arise by any of reactions 13, 14 or 16:

$$\text{Phe.Am} + \text{Tyr.Am} \rightarrow \text{Phe.Tyr.Am} + \text{NH}_3 \quad (12)$$

$$\text{Phe.Tyr.Am} \rightarrow \text{Phe.Tyr} + \text{NH}_3 \quad (13)$$

$$\text{Phe.Tyr.Am} + \text{Tyr} \rightarrow \text{Phe.Tyr} + \text{Tyr.Am} \quad (14)$$

$$2\ \text{Phe.Am} \rightarrow \text{Phe.Phe.Am} + \text{NH}_3 \quad (15)$$

$$\text{Phe.Phe.Am} + \text{Tyr} \rightarrow \text{Phe.Tyr} + \text{Phe.Am} \quad (16)$$

Pepsin, papain, and ficin

Pepsin. Tyrosine amide and tyrosyltyrosine amide were not decomposed by pepsin either at pH 2·2 or at pH 4·2.

Papain. The enzyme was activated by the presence in the reaction mixture of 0·3% (v/v) thioglycollic acid. Tyrosine amide was decomposed more slowly by papain than by chymotrypsin, but gave the same products. Relatively more tyrosyltyrosine was formed at pH 7·0 than at pH 5·0. A relative increase in the extent of transpeptidation with increase in pH has been found for papain by Fruton, Johnston & Fried (1951). Tyrosyltyrosine amide gave only tyrosyltyrosine at pH 7·0, but at pH 5·0 a little tyrosine was also formed. Phenylalanylphenylalanine amide behaved similarly. Tyrosylphenylalanine amide and phenylalanyltyrosine amide each gave only one product at pH 7·0; these were assumed (by analogy with the tyrosyltyrosine amide reaction) to be the corresponding dipeptides.

Ficin. Ficin at pH 4·5 decomposed tyrosine amide and tyrosyltyrosine amide to give the same products as these substrates gave with papain, only more slowly.

DISCUSSION

The action of chymotrypsin on most of these substrates is evidently more complicated than was assumed by Fruton & Bergmann (1942). Measurement of the overall rate of hydrolysis is not adequate to give a correct picture of the course of a reaction when several reactions are proceeding simultaneously. The final products are, however, those expected to arise by hydrolysis of the susceptible bonds.

The transpeptidation reactions catalysed by chymotrypsin are remarkably similar in general outline to those catalysed by trypsin; in both cases cyclic reactions occur. Most of the products initially formed from tyrosine amide by chymotrypsin and from lysine amide by trypsin, arise from the various modes of decomposition of the dipeptide amides. These are in each case formed by transpeptidation of the amino acid amide (reaction 5). The main difference is that trilysine is formed, but trityrosine is not. Similarly, the action of chymotrypsin on dityrosine gives tyrosine and some trityrosine, while the action of trypsin on dilysine gives lysine, trilysine, and some tetralysine. The relative rates of transpeptidation and hydrolytic reactions differ somewhat; while dityrosine is hydrolysed in the presence of chymotrypsin, dilysine in the presence of trypsin reacts only by transpeptidation. The same difference is shown by the formation of a tetrapeptide from dilysine and a tripeptide from dityrosine. Both enzymes catalyse transpeptidations giving intermediates which are broken down by hydrolysis to the amino acids.

SUMMARY

1. The action of chymotrypsin on tyrosine amide, phenylalanine amide, tyrosyltyrosine amide, phenylalanylphenylaline amide, tyrosylphenylalanine amide, phenylalanyltyrosine amide and tyrosyltyrosine has been studied.

2. Hydrolysis is accompanied by transpeptidation in the case of tyrosine amide, phenylalanine amide and tyrosyltyrosine. The products thus formed then break down to give the amino acids as final products.

3. The action of pepsin, papain, and ficin on some of these substrates is also briefly described.

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The Absorption of Methionine by the Skin of the Guinea Pig

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The use of compounds labelled with isotopic sulphur is a well-established method for tracing the origin of the sulphur-containing proteins of hair and skin. Tarver & Schmidt (1939, 1942) fed or injected rats with methionine labelled with ^{35}S and showed that the radioactive sulphur appeared in the hair and tissues as cystine. Du Vigneaud, Kilmer, Rachele & Cohn (1944) labelled methionine with ^{34}S and with ^{13}C in the β and γ positions and fed the amino acid to rats in a diet otherwise deficient of sulphur-containing amino acids. They isolated cystine from the hair of the rats and found by isotopic analysis that about 80% of the sulphur but no significant amount of the carbon had been derived from the methionine. More recently, Williamson & Fromm (1953) have shown that wounded rats, injected with [^{35}S]methionine, accumulate the radioactive sulphur in the wound tissue mainly as cystine, even when the ^{35}S content of the other tissues is decreasing.

The object of the present investigation was to determine whether methionine, rubbed into the skin of the guinea pig, could be absorbed and incorporated into the protein of growing hair. The animals used were all healthy adults, kept on M.R.C. compressed diet no. 18, supplemented by cabbage leaves so that there would be no deficiency of methionine or vitamins essential to the normal growth of the hair. The methionine was labelled with ^{35}S, and evidence was obtained that this was used in forming hair cystine, and appeared in the internal organs even when precautions were taken against the accidental contamination of the food by radioactive material.

EXPERIMENTAL

Mode of application of [^{35}S]*methionine*

The methionine used in this work was DL-[^{35}S]methionine (supplied by The Radiochemical Centre, Amersham, Bucks). It had a specific activity of 0·127 mc/mg. on the initial assay and was applied in the form of an aqueous solution (0·175 mg./ml.). The solution was dispensed from a dropping bottle fitted with a hypodermic needle ground flat at the tip so that one drop of solution (0·0120 $\pm$ 0·0002 ml.) was equivalent to 2·10 μg. of [^{35}S]methionine.

From time to time a drop of the solution was evaporated to dryness on aluminium foil and counted to check the activity. It was found that the observed activity/drop was always within $\pm 2\%$ of the theoretical value calculated from the known half life of ^{35}S. This showed that (i) there was no appreciable loss of material from solution by adsorption on the walls of the container, and (ii) the drop volume remained constant over the period of the investigation.

A single drop of solution delivered in this manner was a convenient unit for application to the guinea pig in the following studies.

In order to compare the relative efficiencies of absorption of the amino acid by intramuscular injection, topical application and feeding, the experimental animals were divided into three groups.

Group I were injected in the thigh with 4 units or 20 units of labelled methionine. Hair clippings were taken at the time of injection and at further weekly intervals until the activity was too small to be measured accurately.

Group II were rubbed daily on the previously prepared area of the back with 1, 4, 10 or 15 units for 4 consecutive days each week and hair clippings taken on the seventh day. This procedure was continued for 4 weeks, after which the application of methionine was discontinued but weekly clippings were taken for a further period until the ^{35}S was eliminated from the growing hair.

Group III were fed with 15 units daily for four consecutive days each week and hair clippings were taken on the seventh day. The feeding was continued for 4 weeks only but clippings were taken every week until no further ^{35}S could be detected.

In all groups the area selected for clipping was marked out on the back of the guinea pig and was, as nearly as could be judged, equivalent to one-tenth of the total hairy surface of the animal. Clippings were taken as close to the skin as possible so that each week's yield represented the total growth of hair over that area for the preceding seven days. It was observed that the growth rate averaged about 0·8 g./week over the whole surface so that the area under investigation usually yielded about 80 mg. weekly, sufficient for the quantitative estimation of ^{35}S content.

The methionine was applied to the animals of group II over the clipped area by measuring the appropriate number of units on to the back of the guinea pig and gently massaging the solution into the skin until moisture was no longer visible. Massage was done with the operator's forefinger encased in a rubber glove, and although there was a certain amount of mechanical loss of radioactive material retained by the glove it was estimated that this was not serious and did not exceed 10% of the amount applied each day. Care was taken that no radioactive material should be allowed to contaminate the food of animals in this group. Excreta and loose hairs were removed from the cages before feeding and the cages were monitored from time to time to check the presence of any stray radioactivity that might otherwise be ingested.

In addition to that area of the back where the labelled methionine was applied, the animals of group II were clipped weekly over an area of equal size on the underbelly, as far as possible from the area of application. This was done because, although all hair samples were given a standard washing treatment before counting, there was the possibility that some of the [^{35}S]methionine might be so firmly adsorbed by the hair that a spuriously high count might be obtained on the samples taken from the back. The clippings from the underbelly, however, would be less liable to contamination, and their ^{35}S content would probably be more representative of the animal as a whole. This was partly confirmed by treating unlabelled hair clippings with various amounts of [^{35}S]methionine and then going through the standard washing treatment to remove the adsorbed amino acid. It was found that hair contaminated at the 10 and 15 units levels sometimes retained as much as 0·1 μg. [^{35}S]methionine/g. whilst the 1 and 4 units levels could retain up to about 0·05 μg./g. Any difference of this order between the activities of hair samples drawn from the back and belly of animals nos. 5–10 during the first 5 weeks could not therefore be interpreted as evidence that the methionine was more readily incorporated into the hair protein immediately adjacent to the area of application.

The measurement of relative activity in guinea pig hair labelled with ^{35}S

For the accurate assay of ^{35}S it is usual to oxidize all sulphur-containing samples to SO_4^{2-} and to precipitate as the barium or benzidine salt before counting under standardized conditions (cf. Henriques, Kistiakowsky, Margnetti & Schneider, 1946). As nearly all the measurements in this investigation were estimates of the relative activity of hair samples, a procedure involving a direct count on the hair itself was adopted; sufficiently reproducible results could rapidly be obtained in this manner without the need for a large number of time-consuming oxidations.

The average length of hair in the weekly clippings taken from each experimental animal was usually about 2 mm. and it was found that a circular pad of hair, formed in a manner analogous to the preparation of the asbestos-fibre pad in a Gooch filter, was uniform enough to give a reproducible count under equivalent geometrical conditions.

The filter assembly used in forming the pad was similar in principle to that described by Comar, Hansard, Hood, Plumlee & Barrentine (1951). The hair clippings were first examined, and any hairs substantially longer than 2 mm. were cut down to this length. The sample was then stirred at 60° for 1 hr. with 1 l. 50% (v/v) aqueous ethanol, filtered, suspended in 0·3 l. 95% ethanol and stirred at 60° for a further 30 min. The ethanolic suspension was filtered on to a 2 cm. Whatman no. 1 paper in the brass filter assembly; the pad of hair was dried under the infrared lamp, mounted on the counting block and placed in a square lead castle (E.R.D. Engineering Co., Ltd., Slough, Bucks) fitted with a type E.H.M. 2 end-window counter (General Electric Co. Ltd.). The counts were made with a Panax Model 44a combined amplifier, scaler, register and power unit (Panax Equipment Ltd., 173, London Road, Mitcham, Surrey).

All observed counts were compared with a [^{14}C]polymethylmethacrylate reference standard and corrected for background (10 counts/min.), resolving time (about 450 μsec.) and radioactive decay, using 87·1 days for the half life of ^{35}S. Where necessary the observed counts were converted into 'infinite thickness' counts by means of a weight-correction curve constructed from the results given in Table 1.

The relative activity of the hair sample could be expressed as a function of the concentration of labelled methionine by hydrolysing hair of known activity and comparing the activity of the hydrolysate with that of a hydrolysate prepared from unlabelled hair containing a known amount of added [^{35}S]methionine.

Thus, 0·3 g. of hair, having an observed relative activity of 510 counts/min. was hydrolysed with 2 ml. 10N-HCl for 12 hr. at 110° and, after removal of excess HCl, the amino acid hydrochloride solution was made up to 5 g. total weight with water; 0·076 g. of this solution, evaporated to dryness

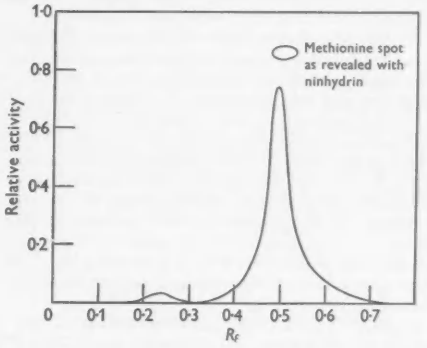

Fig. 1. Distribution of radioactivity along a paper chromatogram of [^{35}S]methionine. For details see text.

Table 1. *Self-absorption correction for three samples of guinea pig hair (see text)*

A		*B*		*C*	
Wt. of hair (mg.)	Counts/min.	Wt. of hair (mg.)	Counts/min.	Wt. of hair (mg.)	Counts/min.
6	14·1	6	38·4	8	125
12	27·6	11	58·2	17	205
17	40·3	19	95·2	23	297
27	55·8	27	131	31	342
37	63·0	45	159	40	376
48	70·0	58	164	50	389
60	72·0	67	169	63	398
73	72·5	85	171	75	405
97	72·4	100	169	90	410

on aluminium foil, had an activity of 204 counts/min. A further 0·3 g. of unlabelled hair was hydrolysed in the same manner in the presence of 0·447 μg. of added [^{35}S]methionine. After removing excess HCl and making up to 5 g., 0·0725 g. of this solution, evaporated to dryness on aluminium foil, had an activity of 307 counts/min.

The weight of dry amino acid hydrochlorides in these activity measurements was of the order 2–3 mg./cm.^{2}, low enough to make the self-absorption factor negligible so that the observed count could be taken as proportional to the ^{35}S content. Then a hair sample of relative activity 1 count/min. would have a [^{35}S]methionine concentration of

$$\frac{204 \times 0{\cdot}0725 \times 0{\cdot}447}{307 \times 0{\cdot}0760 \times 0{\cdot}3 \times 510} = 0{\cdot}00185\ \mu\text{g./g. hair},$$

and from this factor the equivalent weight of [^{35}S]methionine combined in the hair could be calculated for all samples.

Paper chromatography of the amino acids from labelled hair

The combined sulphur in hair is distributed between the methionine and cystine residues of the keratin molecule and, in round figures, the hair hydrolysate contains about 10% of cystine to 1% of methionine (Block & Bolling, 1951). If any of the ^{35}S introduced as methionine is converted into cystine before incorporation in the hair protein, the [^{35}S]cystine should be detectable in the cystine spot after separation of the amino acids by paper chromatography.

A trial paper chromatogram of the original [^{35}S]methionine solution, using approx. 0·1 μg. of amino acid, was cut into small sections and the distribution of radioactivity along the axis was determined by counting the relative activity of each section. It was found that over 90% of the radioactivity was confined to a narrow band (R_F, 0·44–0·54 with a peak at 0·49); about 4% was situated in a secondary band (R_F 0·07–0·29 with a peak at 0·23); the remainder was distributed between these two bands (Fig. 1). The solvent consisted of diethylamine, 10 ml.; *n*-butanol, 50 ml.; ethanol, 50 ml.; water, 25 ml. R_F values of methionine and cystine control spots were 0·5 and 0·2 respectively. Downward displacement on Whatman no. 1 paper was used for all chromatograms.

A sample of labelled hair taken from animal no. 10 at the end of the fifth week, and containing nearly 1 μg. [^{35}S]-methionine/g., was hydrolysed with 10N-HCl in the usual manner and run on a paper chromatogram at the highest practical concentration over a wide front. This was done to increase the amount of radioactive material that could be eluted from each section of the paper and hence increase the sensitivity of the assay. The peak activity, determined after elution from each strip, was found to be concentrated around R_F 0·2; a very small secondary peak was found at R_F 0·6. The R_F values for methionine and cystine hydrochlorides, using the same solvent as before, were 0·6 and 0·2 respectively.

A control hydrolysis of unlabelled hair with added [^{35}S]methionine gave a chromatogram with the main peak at R_F 0·6 and a secondary peak at 0·3. The main peak corresponded to methionine hydrochloride and the secondary peak was probably an oxidation product.

The distribution of activities is shown in Fig. 2.

Precautionary measures taken during topical applications

The possibility that animals receiving topical application might ingest the labelled methionine by licking their hair or by direct absorption through lesions in the skin was given careful attention throughout these experiments. The reasons for accepting these results as evidence of the topical absorption of methionine may be summed up as follows:

(i) Radioactive material was applied only to an area of the back difficult if not impossible for the animal to reach with its mouth or feet. Control animals anointed with a solution of methyl violet in glycerol over an equivalent area could just reach the fringe of the area to lick the coloured glycerol. Here, however, the sensation of moistness on the back and the pleasant taste of the glycerol caused the animals to make a prolonged effort to decontaminate themselves, whereas after the normal methionine rub the back dried almost immediately and the animals exhibited no interest in the contaminated area.

(ii) Each animal had a cage to itself and the cages were frequently cleaned to remove any material that might have been rubbed off the backs of the animals during confinement. The washings were monitored and found to contain very little radioactive material, never more than 5% of the total application.

(iii) In dummy experiments in which the whole of the methionine was applied to the cage itself, the amount of radioactivity in the weekly hair growth was barely detectable, indicating that the animals were not in the habit of licking the wires of the cages.

(iv) None of the experimental animals were ever observed to lick their hair near the area of application.

(v) The animals were fed from clean containers under observation and excess food was removed afterwards to avoid possible contamination with radioactive excreta. The excreta showed some radioactivity after the first week but at such a low level that a very large amount would have to have been eaten to account for the observed activities in the subsequent hair growth.

(vi) When an equivalent dose of radioactive methionine was fed directly to the animals the greater part was elimi-

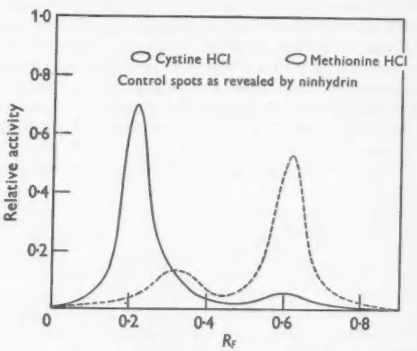

Fig. 2. Distribution of radioactivity along paper chromatograms of hydrolysates of labelled hair (solid line) and of unlabelled hair (hydrolysed in the presence of labelled methionine) (broken line).

nated during the next few weeks and only about 2% of the ^{35}S found its way into the growing hair. This would mean that about half the topically applied methionine would have to have been licked off to give the observed results if none were absorbed through the skin.

(vii) Hair was removed at the beginning of each week by means of electric clippers producing a closely cropped area of skin free from lesions. Direct absorption into the blood stream via cuts or scratches was thereby avoided.

(viii) The rough proportionality of the radioactive content of the hair at the fourth week to the total amount of methionine applied is further evidence in favour of the observed results. Accidental intake by random licking would have produced a less consistent set of results.

RESULTS

The ^{35}S contents of the weekly hair clippings from the fifteen experimental animals, expressed in terms of the original [^{35}S]methionine, are recorded in Tables 2–4, together with an approximate estimate of the percentage recovery of ^{35}S in the hair, based on the assumption that the amount recovered in the clipped area at the end of any one week is one tenth of the total amount combined in the growing hair during that week. Whilst it would have been more satisfactory to have collected the whole hair growth from each animal at the end of every week, it was felt that this treatment was too severe and might have interfered with the health of the guinea pigs. As it was, the animals appeared quite healthy and, with the exception of no. 11 which died at the fourth week, and no. 12 which died during the seventeenth week, all survived the experiments. The lack of hair growth on the clipped underbelly of no. 14 was caused by rubbing against the bottom of the cage; when a different type of cage was used this animal had a normal growth of hair from the eighth week onwards.

DISCUSSION

[^{35}S]Methionine rubbed into the skin of guinea pigs is readily absorbed, and the radiochromatographic analysis of the hair hydrolysate demonstrates quite clearly that the ^{35}S is combined in the hair protein as cystine. This rules out the possibility of chance contamination of hair by physically adsorbed [^{35}S]methionine in any quantity sufficient to confuse the issue. It is interesting to note that, whatever the daily level of methionine applied to the skin (from 1 to 15 units), the overall recovery in the hair is the same in each case, about 1%, and the peak activity of the hair at the end of the fourth week is roughly proportional to the daily level of application. This indicates that the absorption by the skin was directly proportional to the amount applied at these relatively low concentrations. When the same amount was fed (cf. animals nos. 12, 15 with no. 10) about 2·5 times as much ^{35}S was incorporated into the hair. An even higher proportion found its way into the hair protein when a small dose was injected intramuscularly.

On the basis of these experiments there is no reason to suppose that the mechanism of the utilization of the methionine is any different whether the amino acid is introduced into the system by rubbing, injection or *per os*. A large percentage of the topically applied methionine in group I was washed off the subsequent hair clippings, so that although the gross recovery figure was 1% for this series it is likely that the net recovery was of the same order as in group II. Animal no. 9, killed at the end of the fourth week, was dissected and the liver, spleen and heart removed, dried and powdered. The relative activity of these organs was in the ratio 111:42:198, respectively, on a dry matter basis, indicating that

Table 2. *Content of ^{35}S of hair from injected animals*

Animal no.	1	2	3	4
Units injected	4	4	20	20
Total [^{35}S]methionine (μg.)	8·4	8·4	42	42
Weeks	[^{35}S]Methionine equiv. of weekly hair growth (μg./g.)			
1	0·120	0·107	0·333	0·268
2	0·139	0·178	0·568	0·443
3	0·137	0·142	0·305	0·296
4	0·045	0·037	0·137	0·120
5	0·031	0·026	0·109	0·104
6	0·037	0·011	0·070	0·057
7	0·004	0·002	0·043	0·039
8			0·015	0·013
9			0·002	0·001
Total recovery as [^{35}S]-methionine in hair (μg./animal)	0·41	0·40	1·27	1·07
Percentage recovery	4·9	4·8	3·0	2·6

Table 3. *Content of* ^{35}S *of hair from rubbed animals*

Animal no.	5		6		7		8		9		10		13		14	
Units rubbed/day	1		1		4		4		10		10		15		15	
Total [^{35}S]methionine rubbed in 4 weeks (μg.)	34		34		134		134		336		336		441		441	
	[^{35}S]Methionine equiv. of weekly hair growth (μg./g.)															
Weeks	B	U	B	U	B	U	B	U	B	U	B	U	B	U	B	U
1	0·009	0·006	0·006	0·017	0·067	0·011	0·216	0·069	0·383	0·067	0·155	0·080	0·196	0·030	0·213	
2	0·076	0·052	0·043	0·022	0·259	0·205	0·202	0·118	0·500	0·161	0·540	0·352	0·716	0·231	0·740	
3	0·148	0·120	0·091	0·080	0·605	0·203	0·460	0·311	0·718	0·285	0·748	0·774	1·257	0·815	0·980	
4	0·098	0·054	0·080	0·130	0·361	0·426	0·671	0·410	0·918	0·735	0·852	0·845	1·156	0·910	1·340	No
5	0·091	0·113	0·102	0·100	0·374	0·264	0·500	0·367	Killed		0·927	0·963	0·855	0·769	0·686	growth
6	0·041	—	0·052	—	0·278	—	0·420	—	—	—	0·790	—	0·760	0·729	0·832	
7	0·009	—	0·009	—	0·142	—	0·122	—	—	—	0·481	—	0·324	0·296	0·695	
8	0·001	—	0·001	—	0·019	—	0·018	—	—	—	0·209	—	0·318	0·400	0·374	
9	—	—	—	—	0·002	—	0·002	—	—	—	0·046	—	0·242	0·289	0·320	0·294
10	—	—	—	—	—	—	—	—	—	—	0·009	—	0·163	0·262	0·224	0·206
11	—	—	—	—	—	—	—	—	—	—	0·001	—	0·130	0·224	0·157	0·159
12	—	—	—	—	—	—	—	—	—	—	—	—	0·178	0·294	0·100	0·192
13	—	—	—	—	—	—	—	—	—	—	—	—	0·074	0·111	0·072	0·100
14	—	—	—	—	—	—	—	—	—	—	—	—	0·113	0·116	0·068	0·170
15	—	—	—	—	—	—	—	—	—	—	—	—	0·028	0·033	0·072	0·050
16	—	—	—	—	—	—	—	—	—	—	—	—	0·009	0·007	0·033	0·031
17	—	—	—	—	—	—	—	—	—	—	—	—	0·003	0·004	0·010	0·008
18	—	—	—	—	—	—	—	—	—	—	—	—	0·001	0·001	0·003	0·001
Total recovery as [^{35}S]-methionine in hair (μg./animal)	0·35		0·32		1·46		1·78		1·50		3·72		4·82		5·60	
Percentage recovery	1·0		0·9		1·1		1·3		0·5		1·1		1·1		1·3	

B, hair taken from back; U, hair taken from underbelly.

Table 4. *Content of* ^{35}S *of hair from fed animals*

Animal no.	11	12	15
Units fed/day	15	15	15
Total [^{35}S]methionine fed in 4 weeks ($\mu g.$)	441	441	441
	[^{35}S]Methionine equiv. of weekly hair growth ($\mu g./g.$)		
Weeks			
1	0·141	0·282	0·706
2	0·886	0·581	1·85
3	1·48	2·13	2·25
4	Died	3·04	2·85
5	—	2·30	2·42
6	—	1·85	1·29
7	—	1·052	0·820
8	—	0·958	0·760
9	—	0·460	0·346
10	—	0·394	0·425
11	—	0·389	0·292
12	—	0·302	0·220
13	—	0·235	0·185
14	—	0·263	0·111
15	—	0·172	0·075
16	—	0·150	0·027
17	—	Died	0·007
Total recovery as [^{35}S]methionine in hair ($\mu g.$/animal)	2·00	11·64	11·71
Percentage recovery	0·5	2·6	2·7

the ^{35}S was distributed throughout the system in the same manner as if it had been fed or injected (cf. Tarver & Schmidt, 1939). It seems likely that once the methionine reaches the blood stream the demethylation proceeds in the usual manner irrespective of the method of introduction. This does not preclude the possibility that a small proportion of the topically applied methionine might be converted into cystine locally in the root cells, but to demonstrate such a mechanism a more detailed investigation, possibly involving tissue-culture techniques, would be necessary.

SUMMARY

1. [^{35}S]Methionine was applied to the skin of the guinea pig; this resulted in incorporation of ^{35}S in the protein of the growing hair, largely as cystine. This was shown by the distribution of the radioactivity in a paper chromatogram of the hair hydrolysate. The ^{35}S content of the hair was similar whether the specimen was from the area of application of methionine or from a remote area. About 1% of the applied ^{35}S was incorporated in the hair.

2. The amount of ^{35}S absorbed was proportional to the amount rubbed into the skin over the concentration range investigated.

3. If the same amount of [^{35}S]methionine was fed to the guinea pig, about 2·5 times as much ^{35}S was incorporated into the hair.

4. Injection of [^{35}S]methionine intramuscularly was relatively more efficient, as much as 5% of ^{35}S being incorporated in hair protein; larger doses were utilized to a lesser degree.

The author wishes to thank Dr R. H. Marriott for his valuable advice and suggestions; Mr D. M. Brown and Mr J. A. Dimsdale for their care of the animals and practical help; Mr T. L. Hardy for his advice and assistance with the paper chromatography; and Mr E. W. Powell for much useful discussion in the planning period.

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The Effect of Ammonia on the Oxidation of Glucose by *Zygorhyncus moelleri*

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During an investigation of the respiration of filamentous fungi it was observed that when glucose was given to carbohydrate-starved suspensions of several species 2–3 hr. elapsed before the rate of oxygen consumption became constant. This behaviour is markedly different from glucose oxidation by many other micro-organisms under similar conditions, e.g. yeast and the green alga *Chlorella*, in which the addition of glucose to starved cells results in a constant respiratory rate being attained within 10–15 min.

Induction periods have been noticed by other workers in the oxidation of acetate by both yeast and by bacteria. Winzler (1940), investigating acetate oxidation by suspensions of baker's yeast, found that preparations starved for 2 hr. required

7 min. for the oxidation rate to become constant, whereas when starved for 70 hr. the suspension required 70 min., although the final rate of oxygen consumption reached in each case was the same. Barron, Ardao & Hearon (1950) found that *Corynebacterium creatinovorans* grown on acetate and subsequently starved by suspending it in water and aerating with oxygen for 24 hr. showed an induction period on the addition of acetate, and at least 90 min. elapsed before the rate of oxygen uptake became constant. No explanation of these findings were given in either of these two instances.

Lynen (1942) also found an induction period when acetate was added to yeast which had been starved for 15–20 hr. He attributed this to the disappearance during starvation of metabolites necessary for acetate oxidation, and which had to be resynthesized before acetate could be oxidized at a maximal rate.

It was decided to use one of the fungi for an investigation of the factors involved in the appearance of the induction period. The species chosen was *Zygorhyncus moelleri*, a member of the Mucorales. No reference has been found in the literature to previous metabolic studies performed with this organism.

EXPERIMENTAL

Growth. The organism used was *Z. moelleri* (Commonwealth Mycological Institute no. 21994). Considerable difficulty was experienced at first in preparing a suspension of the organism sufficiently homogeneous and finely divided to enable replicate samples to be pipetted into Warburg micro-respirometer flasks. Various methods have been recommended for the preparation of homogeneous fungal suspensions: growth in a Warburg flask itself (Mann, 1944); inoculation of fragmented mycelium into the medium (Dorrell & Page, 1947); inoculation of the medium with a very heavy spore suspension, with shaking during growth (Darby & Goddard, 1950); and aeration of the culture with a forced air stream (Foster, 1949). A discussion of the various methods is also given by Kluyver & Perquin (1933). The following technique was finally adopted: the organism was maintained on potato–glucose–agar slopes, which induced heavy sporulation. Cultures on agar 1–2 weeks old were washed with about 10 ml. of sterile distilled water, accompanied by scraping with a wire loop, and the spore suspension obtained, containing about 4×10^7 spores/ml., was filtered through glass wool in the simple apparatus shown in Fig. 1. The 10 ml. of spore suspension was then inoculated into a 100 ml. Erlenmeyer flask containing 22 ml. of the following medium: glucose, 50 g.; NH_4Cl, 2 g.; KH_2PO_4, 5·4438 g.; N-NaOH, 4·56 ml.; $MgSO_4$, $7H_2O$, 0·1 g.; NaCl, 0·1 g.; Lemco (liver extract) or Marmite (yeast extract), 2 g.; trace element solution, 1 ml.; made up to 1 l. with distilled water. The trace element solution (Horowitz & Beadle, 1943) contained the following trace elements as salts (mg./l.): Boron, 0·01 (as $Na_2B_4O_7$, $10H_2O$); Mo, 0·02 (as $(NH_4)_6Mo_7O_{24}$, $4H_2O$); Fe, 0·2 (as $FeSO_4$, $7H_2O$); Cu, 0·1 (as $CuSO_4$, $5H_2O$); Mn, 0·02 (as $MnSO_4$, $4H_2O$); Zn, 2·0 (as $ZnSO_4$, $7H_2O$). The medium was autoclaved at 15 lb./sq.in. for 15 min.; the pH after autoclaving was 5·2–5·3.

The inoculated medium was shaken for about 18 hr. in a Warburg water bath at 25° (150 oscillations/min.), by which time a good, and very finely divided growth was obtained. The hyphae were centrifuged, washed with 0·066 M phosphate buffer, pH 6·8, and resuspended either in 25 ml. of the growth medium without glucose (if carbohydrate-starved cells were required) or in 25 ml. of 0·066 M phosphate buffer, pH 6·8 (if cells starved of both carbohydrate and nitrogen were required). They were shaken on the Warburg apparatus for a further 24 hr. to complete the starvation process and then centrifuged, washed with buffer, and resuspended in 35 ml. of 0·1 M phosphate buffer, pH 6·8. This suspension contained 1·5–2·0 mg. dry weight of cells/ml. Except where stated to the contrary, the phosphate buffers used in this work were prepared from KH_2PO_4 and KOH. Aseptic precautions were observed during the starvation period but not during the measurement of respiration. Periodic bacteriological tests performed after an experiment have never revealed more than very slight bacterial contamination, even in the presence of both added glucose and NH_4Cl. Even when preparations which had been starved in unsterilized buffer, were incubated with glucose and NH_4Cl it was found that only small numbers of bacteria developed.

Although cell suspensions produced in this way on different occasions were probably not identical with regard to density, homogeneity, or even physiological state, the differences found were apparently quite small.

Manometric methods. Oxygen uptake was measured with Warburg manometers using cups with double side bulbs, and centre wells to which was added 0·2 ml. 15% (w/v) KOH as CO_2 absorbent.

Substrates were added from the side bulb and the main compartment of the cup contained 2·0 ml. of the suspension of hyphae plus sufficient water to bring the final volume of the reaction mixture to 3·0 ml. Organic acids were added as solutions of their sodium salts, and the pH of each solution was adjusted to the required value before use. The gas phase was air save in anaerobic experiments when the manometers were flushed for 10 min. with O_2-free N_2.

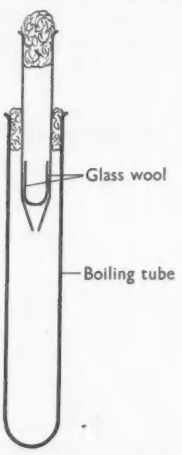

Fig. 1. Apparatus for filtering spore suspension.

Under anaerobic conditions *Z. moelleri* produces CO_2 from glucose and at pH 6·8 a considerable amount of this gas is retained by the buffer. The amount so retained was corrected for by the procedure outlined by Umbreit, Burris & Stauffer (1949), in which α, the solubility coefficient, is replaced by α' in the calculation of the vessel constants. The value of α' was calculated on the basis of the equation:

$$\frac{\alpha'}{\alpha} = \frac{(HCO_3^-) + (CO_2)}{(CO_2)} = [\text{antilog } (pH - 6{\cdot}382)] + 1,$$

where 6·382 is the first dissociation constant for carbonic acid under the conditions of the experiment (Umbreit *et al.* 1949). It should be noted that α' increases rapidly with the pH, and consequently the method is only valid if the pH remains constant throughout the experiment. Further, owing to the rapid increase in the value of α' with pH, it is inadvisable to use this method in buffers more alkaline than pH 7·0.

The respiratory quotient (R.Q.) was measured by the direct method (Dixon, 1943) and the amount of CO_2 formed was corrected for retention by the method outlined above. In four experiments the pH of the medium was measured both at the beginning and at the end of the experiment using a glass electrode and pH meter. The initial values were 6·81, 6·81, 6·80, and 6·82 respectively, and the final values 6·83, 6·79, 6·81, and 6·81 respectively. When the effect of cyanide on the rate of oxygen uptake was investigated, the KOH–KCN mixtures recommended by Robbie (1946) were used for the absorption of CO_2.

Nitrogen analyses. Determinations of the N fractions were carried out as described by Syrett (1953*b*). Briefly, this consisted of centrifuging a sample of the cell suspension and determining the residual free extracellular NH_4^+ in the supernatant in Conway units; the NH_3 released was trapped in 0·05 N-H_2SO_4 in the central compartment, and was subsequently estimated by nesslerization. The centrifuged cells were washed three times with water, and the final centrifugate, still in the centrifuge tubes, immersed in boiling water for 2 min. to kill the cells and rupture the cell membranes. The cell residues were extracted 3 times with water at 80° for 15 min., and the extracts pooled. Total N determinations were performed on the cell residues (insoluble-N fraction) and on the extract (total soluble-N fraction) by the micro-Kjeldahl technique. The NH_3 produced was determined in Conway units followed by nesslerization. The free NH_4^+ in the extract before digestion (free intracellular NH_4^+) was determined directly as described above. All the Conway distillations were allowed to proceed overnight, and all determinations were performed in duplicate.

Dry-weight determinations. The dry weights of mycelial suspensions were measured by drying samples of the suspensions overnight to constant weight at 105°.

RESULTS

Oxidation of glucose. Freshly prepared suspensions of *Z. moelleri* consumed oxygen rapidly, and addition of glucose to such suspensions only resulted in a relatively small increase in the rate of oxygen uptake about double that in the absence of glucose. If the hyphae were starved for 24 hr. by incubating them in a medium containing no carbohydrate but otherwise similar to that used for growth, the endogenous oxygen uptake was reduced. Addition of glucose to suspensions of hyphae which had been so treated resulted in a slow but steady increase in the rate of oxygen uptake which only became constant 2–3 hr. after addition of the substrate. By contrast, suspensions of baker's yeast which had been similarly treated had a lag of some 10–15 min. Fig. 2 shows the results of such an experiment. The final Q_{O_2} (μl. O_2 consumed at s.t.p./mg. dry wt./hr.) was 54. The Q_{O_2} (glucose) varied from preparation to preparation but was usually in the range 50–58. No oxidation products of glucose other than carbon dioxide have as yet been found.

Effect of sodium azide on the rate of oxygen uptake. When a small amount of glucose (6·67 μmoles) was added to suspensions of starved hyphae the extra oxygen consumed was much less than that required for complete oxidation and corresponded to between 16 and 25 % of the theoretical value. This suggested that a considerable amount of the substrate was assimilated. Sodium azide is known to inhibit

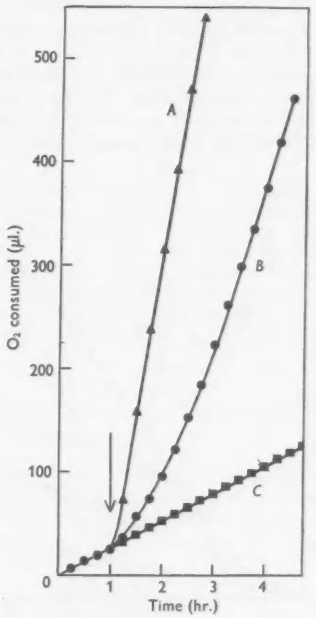

Fig. 2. Comparison of time taken for the rate of O_2 consumption to become constant on the addition of 66·7 μ-moles of glucose to carbohydrate-starved suspensions of baker's yeast and *Z. moelleri*. Arrow indicates addition of glucose. Flasks contained 3·1 mg. dry wt. of *Z. moelleri* or approx. 14 mg. wet wt. of yeast. 0·066 M phosphate buffer, pH 6·8. *A*, yeast; *B*, *Z. moelleri*; *C*, *Z. moelleri* with no added glucose.

oxidative assimilation in other types of cell—see Pickett & Clifton (1943); Winzler (1940); Syrett (1951), and Reiner & Spiegelman (1947)—and Fig. 3 shows the effect of 10^{-3} M sodium azide on the oxidation of glucose by starved suspensions of *Z. moelleri*; it will be noted that the total amount of oxygen consumed was markedly increased but that the inhibitor had no effect on the lag.

Azide usually slightly inhibited the rate of oxygen uptake in the presence of glucose by carbohydrate-starved cells though this did not occur in the experiment recorded in Fig. 3. On the other hand, 10^{-3} M sodium azide increased the rate of endogenous oxygen uptake. If the hyphae were starved by incubation for 24 hr. in phosphate buffer pH 6·8, i.e. in the absence of both carbohydrate and a nitrogen source, then the maximum rate of oxygen uptake in the presence of glucose was unaffected by this concentration of azide. Except where otherwise stated, cells starved in phosphate buffer were used in all subsequent experiments, and such preparations will be referred to as starved cells or starved hyphae.

Effect of ammonium chloride on the oxidation of glucose by starved hyphae. Ammonium chloride increased the rate of oxidation of glucose by suspensions of starved hyphae but had no effect on the endogenous respiration (Fig. 4). The final rate of oxygen uptake attained was the same whether the ammonium chloride was added before, together with, or after the substrate. Ammonium sulphate had the same effect as an equivalent amount of ammonium chloride, indicating that the effect was due to the cation. This stimulatory effect of ammonium salts was inhibited by 10^{-3} M sodium azide (Fig. 4) and by $4{\cdot}6 \times 10^{-4}$ M cyanide, but not by $1{\cdot}67 \times 10^{-4}$ M 2:4-dinitrophenol at pH 6·8.

Fig. 5 shows that the increase in the rate of oxygen uptake is related to the amount of ammonium chloride added over the range 0–5 μmoles/ml. In Fig. 5 the maximum rate of oxygen uptake in the presence of glucose and ammonium chloride, expressed as a percentage of the maximum rate attained in the presence of glucose alone, is plotted against μmoles ammonium chloride added.

If glucose was added to suspensions of starved hyphae in two separate quantities, each of 6·67 μmoles, and the second addition made when the rate of oxygen uptake had fallen to that of the endogenous, there was little or no lag period after the second addition. This was the case whether or not

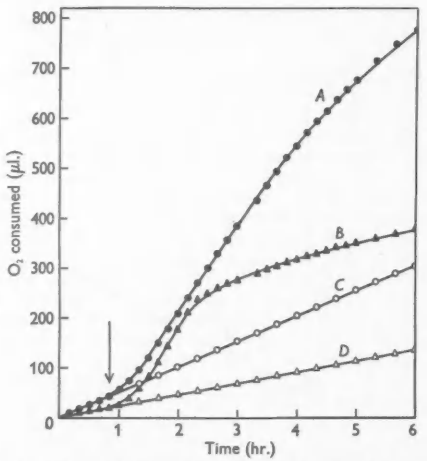

Fig. 3. Effect of NaN_3 on the total O_2 uptake when glucose is oxidized by carbohydrate-starved suspensions of *Z. moelleri*. 6·67 μmoles of glucose added to 6·0 mg. dry wt. of cells/flask as indicated by the arrow. 0·066 M phosphate buffer, pH 6·8. *A*, in the presence of 10^{-3} M-NaN_3; *B*, no NaN_3; *C* and *D*, no glucose, with and without NaN_3 respectively.

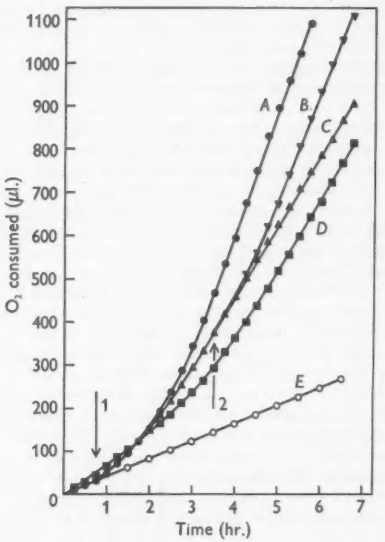

Fig. 4. Effect of NH_4Cl on the rate of oxidation of glucose by carbohydrate- and nitrogen-starved cells in the presence and absence of NaN_3. Glucose concn. 0·022 M in all cases except *E*. Each flask contained 3·2 mg. dry wt. of cells in 0·066 M phosphate buffer, pH 6·8. Where used 9·3 μmoles of NH_4Cl were added/flask. *A*, NH_4Cl present from start; *B*, NH_4Cl added at arrow 2; *C*, no NH_4Cl added. *A*, *B*, and *C* without NaN_3. *D*, as *A*, *B*, and *C* all in the presence of 10^{-3} M-NaN_3: all these curves coincided. *E*, no glucose added, with and without NH_4Cl but without NaN_3. Glucose added to *A*–*D* at arrow 1.

ammonium chloride was present—Fig. 6. It will be noted that, even with these small amounts of glucose, ammonium chloride had a slight stimulatory effect, and this is further borne out by the data presented in Table 1. The rates of oxygen uptake and the total oxygen uptake after several hours in replicate samples of the same batch of cells under the same conditions differed by not more than 2 % from the mean.

That the break in the oxygen uptake curve coincided with the disappearance of glucose from the medium was shown by a semi-quantitative determination of the glucose remaining at the time when the rate of oxygen uptake had returned to the endogenous value. The Ihl-Dische reagent was used and the blue colours produced by samples of the medium were compared by eye with a series of standards of suitable concentration. It was found that when the respiratory rate had fallen to that of the endogenous the glucose concentration in the medium was not more than 10 μg./ml., as compared with an initial concentration of 400 μg./ml.

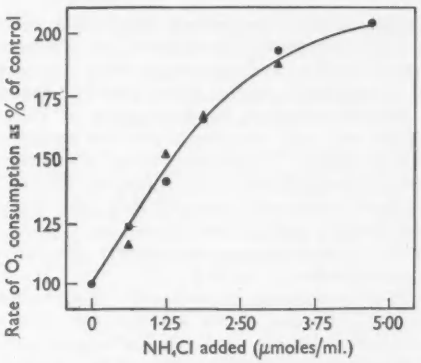

Fig. 5. The effect of the amount of ammonium chloride on the rate of O_2 uptake by carbohydrate- and nitrogen-starved cells oxidizing glucose. Results expressed as percentage of control with no NH_4Cl. Two experiments shown. Glucose concn. 0·022 M in all cases. Each flask contained 3–4 mg. dry wt. of cells in 0·066 M phosphate buffer, pH 6·8.

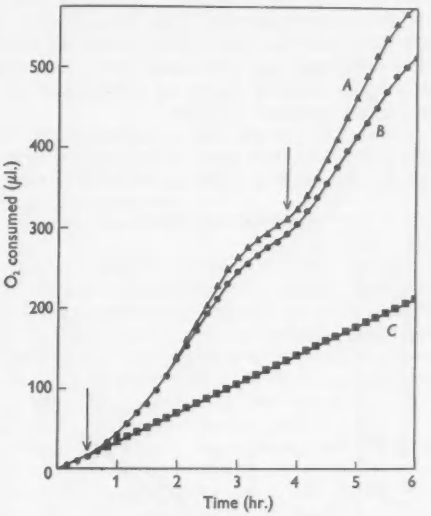

Fig. 6. Addition of two quantities of glucose, each of 6·67 μmoles, to fully starved cells in the presence and absence of added NH_4Cl. Each flask contained 3·7 mg. dry wt. of cells in 0·066 M phosphate buffer, pH 6·8. Glucose added as indicated by the arrows. *A*, in the presence of 9·3 μmoles of NH_4Cl present from the start; *B*, no NH_4Cl; *C*, no glucose added, with and without NH_4Cl.

Table 1. *The reproducibility of the small stimulation by ammonium chloride of the rate of oxygen uptake when the glucose concentration was low*

Three experiments with different cultures were carried out. Each flask contained 3–4 mg. dry wt. of cells; on each separate occasion the dry wt./flask was constant. Where indicated 9·3 μmoles of NH_4Cl were added to the flask; two consecutive quantities, each of 6·67 μmoles of glucose were added to each flask. The medium contained 0·066 M phosphate buffer, pH 6·8. Fig. 6 shows the progress curves of Expt. 1.

Expt.	Rate of O_2 uptake with 1st quantity of glucose (μl./hr./flask)	Ratio: *B*/*A*	Rate of O_2 uptake with 2nd quantity of glucose (μl./hr./flask)	Ratio: *B*/*A*
1. *A* No NH_4Cl	116·0	1·18	114·3	1·37
B With NH_4Cl	136·6		156·6	
2. *A* No NH_4Cl	108·7	1·36	110·1	1·73
B With NH_4Cl	148·0		190·0	
3. *A* No NH_4Cl	120·0	1·32	113·2	1·47
B With NH_4Cl	158·0		165·9	

Effect of ammonium chloride on the fermentation of glucose. Fig. 7 shows that ammonium chloride has no effect on the rate of carbon dioxide formation from glucose under anaerobic conditions; for comparison, the effect of ammonium chloride on the oxidation of glucose by a sample of the same suspension of starved hyphae is shown. It will be noted that a significant period of time elapsed before the rates of both carbon dioxide formation and of oxygen uptake became constant. Thus, despite the fact that under both aerobic and anaerobic conditions there was a lag in the metabolism of glucose, ammonium chloride exerts its stimulating effect only in the presence of oxygen.

Effect of pH *on the rates of oxygen uptake.* The effect of pH on the rates of endogenous oxygen uptake of starved cells and on the rates of oxygen uptake with glucose, both in the presence and in the absence of ammonium chloride, was examined. Phosphate buffers (0·2 M) at a range of pH values were prepared by adjusting solutions of potassium dihydrogen phosphate with hydrochloric acid or sodium hydroxide to the desired pH as measured with a glass electrode. The Warburg flasks were made up as follows: 1·0 ml. buffer, 1·0 ml. of a suspension of starved cells in 0·9 % (w/v) NaCl and glucose and ammonium chloride solutions as required. The maximum constant rates of oxygen uptake were recorded and at the end of the experiment the pH of the medium in each flask was measured using a minature glass electrode assembly capable of measuring pH on about 0·15 ml. of liquid only (Hanes, 1951). The results are given in Table 2.

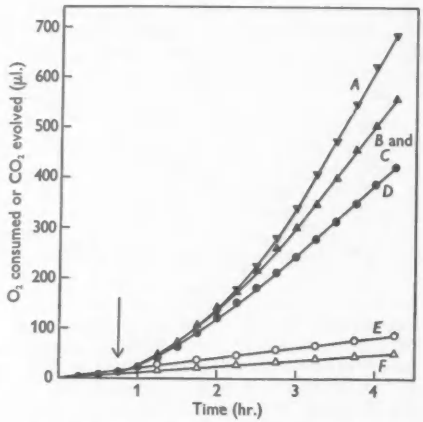

Fig. 7. Effect of NH_4Cl on the rates of O_2 uptake, and of CO_2 evolution under anaerobic conditions, of fully starved cells. *A*, *D*, and *E*, O_2 uptake. *B*, *C*, and *F*, CO_2 evolution. *A* and *B*, NH_4Cl present from start; *C* and *D*, no NH_4Cl; *E* and *F*, no glucose or NH_4Cl. Curves *B* and *C* coincided. 9·3 µmoles of NH_4Cl present in flasks where indicated. Each flask contained 2·8 mg. dry wt. of cells in 0·066 M phosphate buffer, pH 6·8. Glucose added to flasks *A*–*D* at arrow to give a concn. of 0·022 M.

Effect of other nitrogenous compounds on the oxidation of glucose. The effect of the addition of nitrate, nitrite, hydroxylamine, urea, L-aspartic acid, L-asparagine, and L-glutamic acid on the rate of glucose oxidation by suspensions of starved hyphae was next examined, and the results are given in Table 3. L-Glutamic acid and L-asparagine were about as effective as an equivalent amount of ammonia; urea and L-aspartic acid were slightly less effective, and nitrite, nitrate, and hydroxylamine did not increase significantly the rate of oxygen uptake.

Effect of ammonium chloride on the rate of oxidation of other substrates. Table 4 shows the effect of ammonium chloride on the rate of oxygen uptake by suspensions of starved hyphae in the presence of glucose, sucrose, fructose, acetate, and succinate. Since the suspensions did not oxidize succinate at pH 6·8 but did so at pH 3·5, the experiment with succinate recorded in this table was carried out at pH 3·5 (potassium dihydrogen phosphate adjusted to pH 3·5 with hydrochloric acid). Duplicate manometers were set up for each substrate and when the rate of oxygen uptake in the presence of substrate had become constant (120–135 min.) ammonium chloride was added to one of each pair of manometers, the other serving as a control. The experiment was continued until the rates of oxygen

Table 2. *Effect of* pH *on the rate of oxygen uptake under various conditions*

Each flask contained 7·2 mg. dry wt. of cells and 0·066 M phosphate buffer of the pH indicated. Glucose, where present, was at a concn. of 0·022 M. Where indicated 9·3 µmoles of NH_4Cl were added to the flask. The maximum constant rate of O_2 uptake is recorded together with the initial and final pH of the medium in each flask.

	Endogenous		With glucose		With glucose plus NH_4Cl	
Initial pH	Q_{O_2}	Final pH	Q_{O_2}	Final pH	Q_{O_2}	Final pH
3·73	6·3	5·14	33·6	4·93	51·9	3·40
4·98	8·6	5·73	40·0	5·69	56·9	4·76
6·00	9·4	6·24	41·5	6·11	61·7	5·90
7·12	10·3	7·15	31·5	7·15	48·2	7·12
7·98	—	—	14·9	8·17	27·6	7·77
8·81	10·3	8·52	13·8	8·57	23·2	8·02

Table 3. *Stimulation of glucose oxidation by various nitrogenous compounds*

Each flask contained 6·52 mg. dry wt. of cells. The medium contained 0·066 M phosphate buffer, pH 6·8, and 0·022 M glucose in all cases. When the rates of O_2 uptake became constant the nitrogenous substances were added as indicated, the increased constant oxidation rate measured and the time interval between adding the nitrogen and the attainment of the increased oxidation rate noted.

Nitrogenous compound added	Nitrogenous compound added (μmoles)	Nitrogen added (μatoms)	Q_{O_2}	Increase over control (%)	Time interval (min.)
None (no glucose)	0	0	6·4	—	—
None	0	0	26·4	—	—
NH_4Cl	9·3	9·3	53·1	101·1	144
NH_4Cl	4·65	4·65	40·6	54·1	92
KNO_3	9·3	9·3	28·1	6·4	160
$NaNO_2$	9·3	9·3	29·0	9·9	129
NH_2OH, HCl	9·3	9·3	23·2	−12·2	105*
Urea	4·65	9·3	44·6	69·2	120
L-Aspartic acid	9·3	9·3	40·6	54·1	180
L-Glutamic acid	9·3	9·3	49·9†	87·2	—†
L-Asparagine	4·65	9·3	47·2	81·4	123

* Initial inhibition of oxygen uptake followed by subsequent recovery.
† Rate of O_2 uptake still increasing very slowly 285 min. after adding L-glutamic acid.

Table 4. *Effect of ammonium chloride on the oxidation of several substrates*

Rates of oxidation of glucose, sucrose, fructose, acetate, and succinate in the presence and absence of NH_4Cl. Each flask contained 4·65 mg. dry wt. of cells. The medium contained 0·066 M phosphate buffer, pH 6·8 (for succinate oxidation the pH was 3·5). Substrate concn. glucose, fructose, and acetate, 0·02 M; sucrose and succinate, 0·01 M. 9·3 μmoles of NH_4Cl added where indicated.

Substrate	Q_{O_2} No NH_4Cl	Q_{O_2} With NH_4Cl	Increase of Q_{O_2} in presence of NH_4Cl	Percentage increase of Q_{O_2} in presence of NH_4Cl
None	5·0	5·0	0·0	0·0
Glucose	37·0	66·9	29·9	80·8
Sucrose	35·5	60·4	24·9	70·3
Fructose	11·0	12·7	1·7	15·7
Acetate	54·4	66·7	12·3	22·5
Succinate	29·8	31·2	1·4	4·6

uptake had again become constant (a further 90–120 min.). Fructose was oxidized very slowly and ammonium chloride had no effect on the rate of oxygen uptake. The rate of oxygen uptake in the presence of succinate was greater than that in the presence of fructose but it too was unaffected by the addition of ammonium chloride; however, since this experiment was carried out at pH 3·5, this observation may not be significant, although it was noted that ammonium chloride stimulated glucose oxidation at this pH—see Table 2. Glucose and sucrose were oxidized at approximately the same rates, and ammonium chloride had a marked stimulatory effect. Acetate was oxidized the most rapidly of all substrates tested (Q_{O_2} 54·4 as compared with 37·0 for glucose), but even so the rate of oxygen uptake was stimulated to some extent by ammonium chloride.

The fate of added ammonia nitrogen. The fate of ammonia nitrogen added to suspensions of starved hyphae was next examined and the effect of 10^{-3} M sodium azide was also investigated. The suspensions plus additions were shaken in air for 3 hr., at the end of which time the distribution of nitrogen in both cells and suspending fluid was determined. The oxygen uptake was measured manometrically in parallel experiments. The results are given in Table 5. In the absence of azide, 78·8 % of the added ammonia nitrogen disappeared and 54·5 % was accounted for in the insoluble fraction. In the presence of azide assimilation of ammonia nitrogen was almost completely inhibited and 95·4 % of the ammonia nitrogen added was recovered as such. Azide had no effect on the distribution of nitrogen in the cells to which no ammonium chloride had been added. Since azide inhibits both the stimulatory action of ammonium chloride and the assimilation of ammonia nitrogen, it is possible that the stimulatory effect and the assimilatory process are related.

Effect of ammonium chloride on the respiratory quotient. Fig. 8 shows the effect of ammonium chloride on the R.Q. It will be noted that, on addition of ammonium chloride to suspensions of starved hyphae oxidizing glucose, the R.Q. increased from 1·14 to 1·29 and subsequently fell to a value slightly greater than the initial value. This effect is in complete contrast to that observed by Syrett (1953*a*) working with *Chlorella*. In this organism addition of ammonia resulted in a fall in the R.Q. from 1·29 to 0·749 followed by a rise to a value of 1·17 some 90–100 min. later.

Effect of pretreatment with glucose and ammonium chloride. A suspension of starved hyphae was divided into two equal parts, to one of which was added glucose and to the other glucose plus

Table 5. *Distribution of nitrogen in starved cells with and without the addition of an external source of ammonium chloride, in the presence of glucose, and in the presence and absence of sodium azide*

The cells were divided into four equal samples, each containing 24·0 mg. dry wt. of cells (N 8·24 % dry wt.). The cells were incubated in 15 ml. 0·066 M phosphate buffer, pH 6·8, containing 0·022 M glucose together with the following substances: sample 1: none; sample 2: 2·5 mg. of NH_4Cl (654 μg. N); sample 3: 0·001 M-NaN_3; sample 4: 2·5 mg. of NH_4Cl + 0·001 M-NaN_3. The suspensions were shaken in air at 25°. 3 hr. after the addition of the NH_4Cl, samples of each suspension were removed for analysis and the amount of N in each fraction was determined. The O_2 consumption of a sample of each suspension was simultaneously followed.

Fraction	Sample 1 (μg. N)	Sample 2 (μg. N)	Difference between samples 1 and 2 (μg. N)	Difference expressed as % of N added (as NH_4Cl) to sample 2	Sample 3 (μg. N)	Sample 4 (μg. N)	Difference between samples 3 and 4 (μg. N)	Difference expressed as % of N added (as NH_4Cl) to sample 4
Free NH_4^+ in external medium	0·0	75·0	75·0	11·5	0·0	549·8	549·8	84·0
Free NH_4^+ in cells	12·0	19·5	7·5	1·1	11·3	85·5	74·2	11·3
Insoluble N in cells	1545·0	1901·3	356·3	54·5	1477·5	1455·0	−22·5	−3·4
Soluble N in cells	420·0	579·0	159·0	24·3	420·6	447·0	26·4	4·0
Total N	1977·0	2574·8	597·8	91·4	1909·4	2537·3	627·9	95·9
Final constant rate of O_2 uptake (μl./hr./flask)	187	330	—	—	191	207	—	—

Rate of O_2 uptake with no added NH_4Cl, glucose or NaN_3: 54 μl./hr./flask.

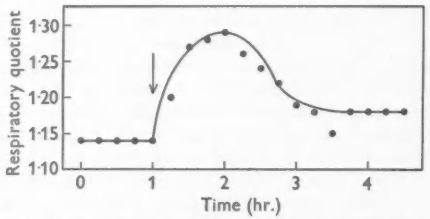

Fig. 8. Change of R.Q. following addition of NH_4Cl to fully starved cells oxidizing glucose. 9·3 μmoles of NH_4Cl added to 3·9 mg. dry wt. of cells in 0·066 M phosphate buffer: original pH 5·26; final pH 5·17. NH_4Cl added at arrow; 0·022 M glucose present from start.

ammonium chloride. Both samples were shaken in air for 3 hr. at 25°, at the end of which time the hyphae were centrifuged off, washed and suspended in water. The ability of both suspensions to oxidize glucose, acetate, ethanol and succinate was then examined. In the case of succinate the pH of the medium was buffered at pH 3·5 as described above; the other three substrates were tested at pH 6·8 in the usual way. The results are given in Table 6. It will be seen that when glucose and acetate were the substrates, the rates of oxygen uptake were greater after pretreatment with glucose and ammonium chloride than after pretreatment with glucose alone. Ethanol oxidation showed a qualitatively similar response, though the effect was smaller; the rate of oxidation of succinate was similar after both types of treatment. It will also be seen that the dry weight of the hyphae was increased by both treatments.

Table 6. *Effect of treating cells with ammonium chloride and glucose on the dry weight and the rates of oxygen uptake with several substrates*

96·8 mg. dry wt. of cells were divided into two samples. Each sample was suspended in 15 ml. of 0·066 M phosphate buffer, pH 6·8, and received 333·3 μmoles of glucose; sample 2 also received 77·5 μmoles of NH_4Cl. Both samples were shaken in air at 25° for 3 hr. The cells were then centrifuged, washed, and each sample suspended in 15 ml. of water. 1 ml. of cell suspension was used in each Warburg flask, which also contained 0·066 M phosphate buffer, pH 6·8 (for succinate oxidation the pH was 3·5) and the substrate at a final concn. of 0·022 M. Dry weight determinations were performed on the original cells before treatment, and on each of the two samples after treatment with glucose with or without NH_4Cl.

	Q_{O_2}*		
Substrate	Sample 1. Cells treated with glucose only	Sample 2. Cells treated with glucose + NH_4Cl	Percentage increase in sample 2 over sample 1
Glucose	25·7	36·5	42·2
Acetate	42·4	50·5	19·1
Ethanol	16·7	20·4	22·3
Succinate	12·4	12·7	2·5

Dry weight of cells in equivalent volumes of suspension (mg.):

Before treatment	16·1
After glucose alone (sample 1)	18·5
After glucose + NH_4Cl (sample 2)	19·4

* Calculated on dry-weight basis of original untreated cells.

DISCUSSION

Washed suspensions of freshly harvested *Z. moelleri* have a high endogenous respiration and addition of glucose to such suspensions only caused a relatively small increase in the rate of oxygen uptake. On the other hand, if the suspensions were starved for 24 hr. in phosphate buffer at pH 6·8, the endogenous respiration was reduced and addition of glucose resulted in a steadily increasing rate of oxygen uptake which became constant after a period of 2–3 hr. This phenomenon is not peculiar to the aerobic metabolism of glucose by suspensions of starved hyphae, since a similar lag was observed when such suspensions metabolized glucose anaerobically. The fact that the lags observed under both aerobic and anaerobic conditions were of similar duration suggests that a common factor, or factors, are involved.

Sodium azide is known to inhibit oxidative assimilation, oxidative phosphorylation and adaptive enzyme formation (Clifton, 1946; Spiegelman, 1947; Spiegelman, Kamen & Sussman, 1948; Winzler, 1940, 1944; Winzler, Burk & du Vigneaud, 1944). This inhibitor at a concentration of 10^{-3} M had no effect on the time course of oxygen uptake in the presence of glucose. Consequently, this increase in the rate of oxygen uptake is not due to adaptive enzyme formation, and the cause of it remains unknown. But azide was not without action on the metabolism of glucose. It was found that it increased the total amount of oxygen consumed. In the absence of azide the total oxygen uptake was 16–25 % of that required for the complete oxidation of the glucose added to carbon dioxide and water, whereas in the presence of azide almost the theoretical uptake was observed, suggesting that this mould has very great assimilatory powers.

When ammonium chloride was added to a suspension of starved hyphae oxidizing glucose the final rate of oxygen uptake was somewhat greater than that obtained in the presence of glucose alone. But ammonium chloride had no effect on the endogenous rate of oxygen uptake; nor was the rate of anaerobic metabolism of glucose influenced by this compound. L-Glutamic acid, L-asparagine, and to a lesser extent, L-aspartic acid and urea showed effects similar to that of ammonium chloride on the rate of glucose oxidation; but nitrate, nitrite, and hydroxylamine had no action on this process. The effect was not specific for glucose; the rates of oxidation of sucrose, and, to a lesser extent, acetate were also increased by ammonium chloride. On the other hand, the rates of oxygen uptake in the presence of fructose and succinate were unaffected by this treatment.

Sodium azide at a final concentration of 10^{-3} M completely abolished the effect of ammonium ions on the oxidation of glucose. Azide is known to inhibit synthetic processes and it would seem reasonable to suggest that the effect of ammonium ions is associated with the synthesis of some nitrogenous compound or compounds, possibly protein in nature, which are essential for the oxidation of the substrate. Analysis showed that the ammonium chloride added to suspensions oxidizing glucose almost completely disappeared, and that its disappearance was accounted for by an increase in the nitrogenous constituents of the hyphae. More than half of the ammonia nitrogen disappearing was accounted for by an increase in the nitrogen content of the insoluble fraction, an observation in keeping with the view that the action of ammonium chloride is associated with protein synthesis. It was particularly significant, therefore, that azide was found to inhibit not only the effect of ammonium chloride on respiration but also the assimilation of ammonia nitrogen. It is noteworthy in this connexion that Yamagutchi & Matsuzaki (1952), working on the adaptation of *Pseudomonas aeruginosa* to propionate, found that the developments of enzyme activity was stimulated by the addition of ammonium sulphate; below 4×10^{-4} M there was practically a linear relationship between ammonium sulphate added and the maximum level of enzyme activity attained. Endogenous respiration was practically unaffected by ammonium sulphate. Spiegelman & Dunn (1947) found that the presence of an external nitrogen source stimulated the rate of adaptation and considerably raised the attainable level of enzyme activity in the adaptation of yeast to galactose and maltose fermentation. This stimulation was absent if the cells were unable to assimilate the added nitrogen, e.g. in the presence of azide.

It is not possible as yet to say whether general protein synthesis occurs in the presence of glucose and ammonium ions, or whether the effect is specific for those enzymes concerned with the oxidation of glucose. The results given in Table 6 show that preincubation of hyphae with glucose and ammonium chloride results in an increased rate of oxidation of both glucose and acetate, suggesting that the amounts of the enzymes concerned with the oxidation of these two compounds are increased. This must remain a suggestion until such time as methods have been perfected for the quantitative estimation of the amounts of these enzymes within the hyphae.

SUMMARY

1. *Zygorhyncus moelleri* was grown in a glucose–ammonium chloride medium and then starved for 24 hr. in a carbohydrate-free medium. When glucose was added to the starved cells an induction period of 2–3 hr. was observed before the rate of oxygen uptake rose to a constant maximum value.

2. In the presence of azide the final rate of oxygen uptake was somewhat lower than in its absence, although the length of the induction period was not greatly affected. These cells were not nitrogen-starved.

3. The respiration of suspensions starved of both carbohydrate and nitrogen was not inhibited by azide, but was greatly stimulated by small quantities of ammonium chloride. This stimulation was abolished by suitable concentrations of azide and cyanide.

4. Neither the endogenous respiration nor the fermentation of glucose under anaerobic conditions was affected by added ammonium chloride.

5. L-Glutamic acid, L-aspartic acid, L-asparagine, and urea stimulated glucose oxidation, though none so effectively as ammonia; nitrate, nitrite, and hydroxylamine were inactive.

6. The oxidation rates of sucrose, ethanol, acetate and succinate were also stimulated to varying degrees by ammonia, but the stimulation was much weaker in most cases than with glucose; only with sucrose did the stimulation approach that with glucose.

7. In the presence of glucose the ammonia was bound, and after 3 hr. appeared mainly in the insoluble nitrogen fraction; azide inhibited this binding of ammonia.

8. These results are discussed and it is suggested that enzyme adaptation is a possible interpretation of these phenomena consistent with the observed facts.

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Studies on Yeast Metabolism

3. THE INTRACELLULAR LEVEL OF PYRUVATE DURING YEAST FERMENTATION

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The rate of carbon dioxide production during alcoholic fermentation is a direct measure of the velocity of the yeast carboxylase system, i.e. of the reaction:

$$\text{pyruvate} + H^+ \rightarrow \text{acetaldehyde} + CO_2.$$

This is irreversible (Vennesland, 1951); thus, in the steady state during fermentation, the rate of pyruvate decarboxylation is likely to be determined by the substrate concentration at the enzyme surface. Unfortunately, this quantity cannot be directly determined. The amount of pyruvate in unit weight of yeast at any stage during fermentation is, in principle, determinable. This intracellular level of pyruvate may or may not be simply related to the kinetically important concentration at the site of carboxylase action. If it is, then its measurement at different fermentation rates should give information about the kinetics of yeast carboxylase *in vivo*.

Extracellular pyruvate, appearing in the medium during fermentation, has been frequently studied; recently, for example, by Wang, Labbe, Christensen & Cheldelin (1952). A short report on the intracellular level has appeared (Brady, 1950).

METHODS

Fermentation was carried out at 30°, using 5 g. (wet wt.) of D.C.L. baker's yeast/100 ml.; the suspension was stirred by a stream of gas, usually N_2. Yeast was taken as required from a 500 g. block, wrapped in a plastic bag, and stored in the refrigerator. Samples taken daily over 3 weeks from yeast stored in this way varied little in performance, as shown by fermentation rate or levels of pyruvate and inorganic phosphate. In consequence, such determinations are strictly comparable although fermentation rate and pyruvate levels were not measured simultaneously, but in separate experiments.

Determination of fermentation rate

The average fermentation rate over an interval of (usually) 15 min. was determined after absorption of CO_2 in 50 ml. approximately N-NaOH, by titration of precipitated $BaCO_3$. Absorption towers were connected to the vacuum supply in such a way that connexion or disconnexion did not affect the gas pressure in the fermentation vessel. The method proved sensitive and reproducible: duplicate determinations of the rate at a definite time after initiation of fermentation differed by about 2%.

Maximum fermentation rate was not attained until 35 min. or so after addition of glucose: thereafter, the rate gradually declined. Of the constituents added to the medium, most had only a minor effect. Phosphate caused a more rapid decline in later stages of glucose fermentation, which could be partially counteracted by the addition of magnesium and ammonium salts. In a medium consisting of 0·2 M glucose, succinate buffer pH 4·1 (0·05 M succinic acid + 0·025 N-NaOH), 0·01 M-KH_2PO_4, 0·01 M-$(NH_4)_2SO_4$, 0·0025 M-$MgSO_4$, 0·001 M-$CaCl_2$ and 0·001 M-$ZnSO_4$, the fermentation rate was almost constant after 30 min., and this medium was consequently particularly suitable for kinetic studies.

Determination of extracellular pyruvate

A sample (about 3 ml.) of yeast suspension was rapidly transferred to a centrifuge tube and centrifuged 3 min. at 3000 rev. min.$^{-1}$ About 30 sec. elapsed between the time of pipetting the sample, which was recorded, and reaching full speed in the centrifuge. A suitable portion of supernatant was delivered into trichloroacetic acid (TCA), the final solution being 4 ml., 0·5 M in TCA. This was kept in an ice-bath for analysis of pyruvate by the method of Friedemann & Haugen (1943).

Determination of total pyruvate

(1) TCA extraction: 2 ml. yeast suspension were pipetted into an equal volume of chilled M-TCA. After 10–15 min. in an ice-bath the yeast was centrifuged down and a portion of the supernatant made up to 4 ml. (0·5 M in TCA) for analysis. (2) Perchloric acid extraction was similarly performed. (3) Hot-water extraction: the yeast suspension was delivered into 2·5 times its volume of water at 100° (boiling water bath), and after 5 min. cooled, centrifuged and treated as (1).

As Table 1 shows, methods (1) and (3) gave concordant results, whereas method (2) gave values for total pyruvate similar to the extracellular values. (In a fermentation using phosphate buffer as medium, perchloric acid extracts contained pyruvate in amount intermediate between the extracellular value and that in TCA extracts.) Perchloric acid, which is highly dissociated, probably does not enter the cell and destroy enzyme action sufficiently rapidly. Unless otherwise stated, 'total pyruvate' means the value obtained by TCA extraction.

Calculation of intracellular pyruvate

All pyruvate values are expressed per g. fresh weight of yeast; in the case of extracellular pyruvate this means the amount in that volume of medium which contained 1 g. yeast. The volume occupied by the yeast cells in a suspension was taken to be three-quarters of the centrifuged cell volume, as shown by Conway & Downey (1950), whose haematocrit technique was used. Little variation in the yeast cell volume was found in the different fermentation media employed, and it was considered sufficiently accurate to take the volume of extracellular medium in 100 ml. suspension containing 5 g. yeast as 96·0 ml. No correction was applied to the total pyruvate for the volume of the yeast solids, as such correction was within the analytical error. The difference between the total pyruvate and the extracellular pyruvate, as μmoles/g. yeast, at a given time, is taken as the intracellular pyruvate.

Table 1. *Pyruvate extracted from a fermenting yeast suspension by different methods*

Suspension: 5 g. baker's yeast + 10 m-moles glucose per 100 ml. (1), (2), (3) refer to experiments made at 3-day intervals.

	Total pyruvate (μmoles/g.)					
		Trichloroacetic acid extract		Perchloric acid extract		
Time (min.)	Hot-water extract					Extracellular pyruvate (μmoles/g.)
1	1·0 (2)	0·7 (1)	—	0·1 (1)	0·1 (2)	—
5	3·4	3·0	—	0·8	0·9	—
10	5·8	5·2	5·8 (3)	1·9	2·1	2·0 (3)
20	8·4	8·6	8·9	3·7	4·1	4·1
30	11·3	11·4	12·2	7·0	7·4	5·4
40	—	—	13·7	—	—	6·7

Ion-exchange chromatography

The nature of the material determined in the colorimetric analysis of pyruvate was examined by anion-exchange chromatography on a 2·5 cm. × 1 cm.² Dowex-1 column (acetate form), using 0·1 N acetic acid + 0·05 M sodium acetate as the eluting solvent. From a yeast suspension which had fermented 0·1 M glucose for 30 min. were prepared: (i) a centrifugate, containing the extracellular pyruvate (6·7 μmoles/g.), (ii) a hot-water extract (total pyruvate, 12·1 μmoles/g.), whilst in a recovery experiment (iii) a known amount of pyruvic acid was added to a centrifugate. Material run on to the Dowex column corresponded to 1·25 g. yeast; the eluate was collected in 25 ml. fractions, which were analysed for pyruvate. In each case, the elution curve suggested the presence of only a single compound. 84–100% of the material added was recovered. It is therefore probable that the bulk of the material reacting in the colorimetric method was, in fact, pyruvate. Ion exchange was also employed to obtain, from sodium pyruvate prepared according to Robertson (1942), a solution of pyruvic acid for use as a standard in the colorimetric analysis. Standardization of the solution was by an iodometric method (Wendel, 1931), checked by a gravimetric method using the 2:4-dinitrophenylhydrazone.

RESULTS

Intracellular pyruvate and fermentation rate

Buffers, when used, contained 5 m-moles succinic acid/100 ml. suspension, and either 2·5 m-moles NaOH (pH 4·1) or 7·5 m-moles NaOH (pH 5·5). All constituents except sugar were brought to the temperature of the thermostatic bath, the suspension being stirred by a stream of nitrogen. Fermentation was initiated by adding the required amount of sugar, as a M solution of glucose.

With no sugar present, the intracellular pyruvate (succinate buffer, pH 5·5) was 0·3 μmole/g., rising only to 0·4 after 30 min. incubation. The interpretation of such small figures as pyruvate is doubtful (Brady, 1950). The close relation between intracellular pyruvate and fermentation rate is shown in Fig. 1, which represents the results of an experiment in which 10 m-moles glucose were completely fermented. Inorganic phosphate (intracellular, the medium gave only trace values throughout) was determined, using an adaptation of the method of Berenblum & Chain (1938), applied to TCA extracts obtained as described under 'Methods'. The results of two separate experiments (Fig. 1) show that the onset of phosphorylation processes was considerably more rapid than the overall fermentation, and suggest that rate control in the first 30 min. of this fermentation was due to enzyme systems intermediate between fructose 1:6-diphosphate and pyruvate. Fig. 2 shows that exhaustion of external glucose (determined, after centrifuging off the yeast, by the method of Heidt, Southam, Benedict & Smith, 1949) brought about a rapid fall in intracellular pyruvate, the level of which, however, was still appreciable long after the disappearance of glucose. The excretion of pyruvate into the medium also stopped when all the glucose had disappeared, and the level of pyruvate outside the cell began slowly to decline.

The quantitative relation between fermentation rate and intracellular pyruvate was examined by making six sets of measurements of rate and pyruvate level, at 15 or 30 min. intervals, on each of six anaerobic fermentations. The same master sample of yeast was used in the first five experiments: the conditions of fermentation were not, however, the same. Thus thirty-six determinations of rate and intracellular pyruvate were obtained, the rate varying from 10 to 53 μmoles/min./g. and the

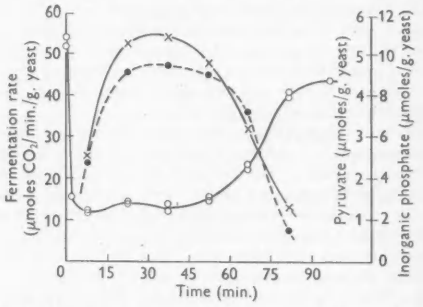

Fig. 1. Intracellular pyruvate (×), fermentation rate (●), and inorganic phosphate (○). 5 g. yeast, 10 m-moles glucose/100 ml., succinate buffer pH 4·1.

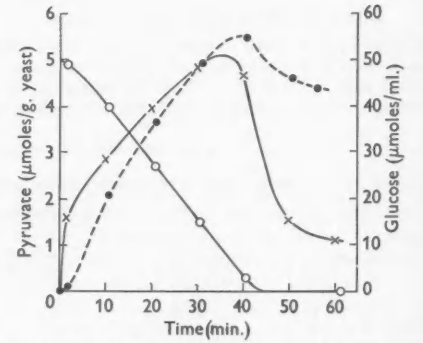

Fig. 2. Intracellular pyruvate (×), extracellular pyruvate (●), and glucose uptake (○). 5 g. yeast, 5 m-moles glucose/100 ml.

pyruvate from 1 to 13 μmoles/g. Some of these measurements were, however, discarded: those made 82·5 min. after initiation of glucose fermentation were unsatisfactory because of the high extracellular pyruvate (for then determination of the intracellular level by difference became inaccurate), while at 7·5 min. the pyruvate measurements were highly accurate but the rate measurements were uncertain, due to rapid variation of rate with time in the initial stage of fermentation.

In four experiments (*a*)–(*d*), glucose was the substrate. The composition of the medium, and the maximum rate (as μmoles CO_2/min./g.) recorded, were as follows: (*a*), no addition, 45; (*b*) constant-rate medium described under 'Methods', 53; (*c*) as (*b*), but KCl replaced KH_2PO_4 (phosphate-free medium), 47; (*d*) succinate buffer pH 4·1, 0·001 M sodium arsenate, 16. Arsenate is particularly suitable as an inhibitor, since the fermentation rate was found to be insensitive to changes in arsenate concentration between 10^{-4} and 5×10^{-3} M, and the effect of the inhibitor was therefore highly reproducible. Low values of rate and pyruvate were recorded also in experiment (*e*), in which the yeast fermented maltose in succinate buffer. Pyruvate varied from 1·2 to 2·3 μmoles/g. whilst the rate changed from 10 to 21 μmoles CO_2/min./g. In maltose fermentation the rate increased slowly with time, owing to formation of the adaptive enzyme maltase.

Examination of the results showed that the intracellular pyruvate increased more rapidly than fermentation rate. This is reminiscent of the effect produced when an enzyme becomes saturated by its substrate (see, for example, Van Slyke, 1942). Fig. 3 shows the application of the simple Michaelis-Menten equation to the results of Expts. (*a*)–(*d*) (glucose fermentation). The form

$$S/v = S/V_{max.} + K_m/V_{max.}$$

is used, as this emphasizes high substrate values (Hofstee, 1952) which form the bulk of the data. The maximum fermentation rate, $V_{max.}$, derived from the slope of the straight line fitted to the plot of S/v against S by the method of least squares, is 65 μmoles CO_2/min./g. yeast; half-maximum rate is produced at an intracellular pyruvate level of $S = K_m = 3{\cdot}1$ μmoles/g.

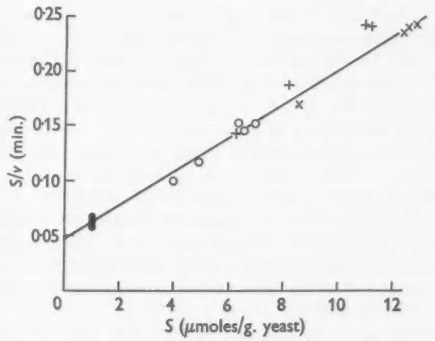

Fig. 3. Michaelis plot of S/v against S. S = intracellular pyruvate (μmoles/g. yeast); v = fermentation rate (μmoles CO_2/min./g. yeast). ○, data of Expt. (*a*) (see text); ×, Expt. (*b*); +, Expt. (*c*); ●, Expt. (*d*).

Experiment (*f*), which used a different sample of D.C.L. baker's yeast, showed that the time-dependent rate of maltose fermentation enables the values of $V_{max.}$ and K_m to be derived from measurements made between 60 and 240 min. in a single fermentation, though less reliably, since the maximum rate obtained is less than that with glucose in the same medium. Here $V_{max.} = 63$ μmoles CO_2/min./g.; $K_m = 2{\cdot}8$ μmoles pyruvate/g.

Pyruvate levels in various fermentation systems

Once the relation between the intracellular level of pyruvate and the fermentation rate has been established, the fermentation rate, or what amounts to the same thing, the velocity of the yeast carboxylase system, may be derived from pyruvate measurements. During the present investigation, pyruvate levels during the first 30 min. of fermentation were measured in a number of systems; usually 20 ml. of the fermenting suspension contained in a 1 in. diameter Pyrex tube were stirred (at 30°) by bubbling gas (N_2, O_2, CO_2). The rate of CO_2 production was not measured, though an approximate idea of it could be derived from the rate of glucose uptake. Rate measurements made by the CO_2 production method described are averages over at least 10 min., and are valueless when the rate is rapidly changing with time, as it does during the first 30 min. of anaerobic fermentation, with the yeast we have used. Pyruvate measurements can be made, however, as early as 1 min. after mixing the yeast and glucose. An expected error in the extracellular value, due to the time taken in centrifuging, did not arise—since the extracellular pyruvate was in fact negligible. At this time, in one experiment, the intracellular pyruvate had increased from 0·7 μmole/g. before addition of glucose to 1·5. At 5 min. the value was 4, after which the level rose by about 1 μmole/g. every 5 min. Since the rate/pyruvate relation is hyperbolic, these figures suggest an initially rapid rise in rate, the acceleration decreasing with time.

An experiment in which glucose was metabolized aerobically is interesting. If the hyperbolic relation between pyruvate level and the velocity of the carboxylase system, derived from studies of

anaerobic fermentation, is assumed to apply here, then the pyruvate level of 1·6 μmoles/g. observed corresponds to a carboxylase rate of about 20 μmoles CO_2/min./g., or 0·3 of the rate in anaerobic fermentation. The glucose uptake was about 0·35 of that in the anaerobic system. This strongly suggests that, under the experimental conditions employed, glucose was metabolized by the anaerobic system as far as acetaldehyde (cf. Weinhouse, Millington & Lewis, 1948). Further, the suggestion of Myrbäck & Vallin (1944) that the Pasteur effect in yeast is due to inactivation (as the disulphide) of cocarboxylase receives no support from this experiment, for no accumulation of pyruvate was demonstrable. Replacing nitrogen by CO_2 lowered the intracellular pyruvate during the first 30 min. of fermentation. Adding bicarbonate to give a higher pH increased the extracellular pyruvate production, though the intracellular levels were not affected proportionately.

The strain of *Saccharomyces cerevisiae* used in the manufacture of D.C.L. baker's yeast was, in some preliminary experiments, grown in the laboratory under sterile conditions. Eighteen-hour cultures were employed, grown at 30° in the synthetic medium of Olson & Johnson (1949). The intracellular pyruvate level (1–2 μmoles/g.) was considerably lower, during fermentation, than in comparable experiments using pressed yeast; and this was irrespective of whether the medium contained thiamine or not. This suggests (1) that the yeast strain is capable of cocarboxylase synthesis without an external supply of thiamine, and (2) that the high intracellular pyruvate level of pressed yeast (or at least of the particular type we have used) is not a feature of the strain of organism, but due to the method of manufacture.

DISCUSSION

We have shown that the velocity of the carboxylase system in the living yeast cell (which in anaerobic fermentation is identical with the rate of carbon dioxide production) depends on the intracellular pyruvate level in the same way as, *in vitro*, the velocity of a simple enzyme reaction depends on substrate concentration. This relation may be treated as a purely empirical one. Even so, the results demonstrate that in a favourable case the methods of enzyme kinetics may be applicable to the living cell. Again, the calculation of carboxylase activity from pyruvate measurement, as applied for example to the preliminary experiment on aerobic glucose metabolism reported in the previous section, does not necessarily depend on a particular interpretation of the carboxylase–pyruvate relation. The apparent 'saturation' of the yeast carboxylase with pyruvate at higher fermentation rates may be interpreted in several ways. For example, the carboxylase may be inhibited by some substance (e.g. acetaldehyde) which increases in concentration as fermentation rate increases. The simplest hypothesis is, however, that referred to in the Introduction; namely, that the intracellular pyruvate, as defined in the section on Methods, is a direct measure of pyruvate concentration at the site of carboxylase action. It is important to note that the accuracy of the data obtained does not permit any deduction based on the exact form of the mathematical relation between fermentation rate and pyruvate level.

The spatial distribution of the carboxylase system in the yeast cell is not known. Chantrenne (1944) found carboxylase activity in yeast extracts was not associated with particulate material. However, according to Michaelis (1949), the kinetics of an enzyme system are similar whether the enzyme is in homogeneous solution or in particulate form; consequently, it is not the distribution of carboxylase within the yeast cell that is important, but the distribution of the substrate pyruvate. If this is assumed to be equally distributed throughout the cell, i.e. diffusion rates are taken to be rapid in comparison with the rate of enzyme action, then the intracellular pyruvate level, as μmoles/g. yeast, may be transformed into substrate concentration if the intracellular water content of the yeast is known. According to Lindenberg, Monique & Guillemet (1949) baker's yeast dispersed in water contains 66% intracellular water, which falls to 63·5% when the yeast is suspended in 0·1 M-NaCl. Thus, on the assumption that we have actually measured substrate concentration and carboxylase velocity, the results may be briefly stated as follows: for the yeast used in the series of experiments (*a*)–(*e*) described above the *in vivo* activity (maximum velocity) of carboxylase was 65 μmoles pyruvate (or CO_2)/min./g. yeast, and the Michaelis constant 3 μmoles/g. or about 4×10^{-3} M pyruvate. [Green, Herbert & Subrahmanyan (1941) found the Michaelis constant of purified yeast carboxylase in citrate buffer, pH 6, to be 3×10^{-2} M. An earlier report (Hägglund & Rosenquist, 1927) stated that pyruvate concentration had no effect on the velocity of CO_2 production by a crude yeast preparation when varied between 0·03 and 0·50 M, but at 0·01 M activity was markedly decreased. Wheat germ carboxylase, which has been obtained in a highly purified state by Singer & Pensky (1952), is half-saturated at $3{\cdot}6 \times 10^{-3}$ M pyruvate (succinate buffer pH 6, 30°).]

The question of whether the rate of alcoholic fermentation can be said to be 'limited' by one or other enzyme system has been frequently discussed. With such a complex process as fermentation by living yeast, in which so many factors are beyond

experimental control, no general satisfactory definition of 'limiting enzyme' can be made. In the case of an enzyme catalysing the irreversible decomposition of a single substrate, it will clearly limit the overall rate of fermentation when it is saturated with respect to its substrate. When the yeast we have studied ferments excess glucose at 30° carboxylase very nearly meets this requirement: according to the simple interpretation of the results we have suggested, this enzyme system is about 80% saturated by 12 μmoles pyruvate/g. yeast. It should be noted that this does not imply that raising the activity of the carboxylase system would necessarily result in an increased fermentation rate: what is implied, however, is that no substantial rate increase is possible unless the activity of intracellular carboxylase is increased through synthesis of specific protein, or of coenzyme, or even possibly by alteration of physico-chemical properties of the enzyme milieu, such as pH.

SUMMARY

1. The intracellular pyruvate level of yeast has been measured as the difference between the total pyruvate extracted from a suspension by trichloroacetic acid and that excreted into the medium.

2. The relation between fermentation rate and intracellular pyruvate resembles that between the velocity of an enzyme reaction and substrate concentration.

3. The velocity of the carboxylase system in metabolizing yeast suspensions may therefore be deduced from pyruvate measurement when direct measurement by determination of carbon dioxide output is not feasible.

4. A possible interpretation of the results as a direct determination of the kinetics of the carboxylase system *in vivo* is given.

5. The limitation of fermentation rate, in the yeast studied, by carboxylase is discussed.

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Studies on Yeast Metabolism

4. THE EFFECT OF THIAMINE ON YEAST FERMENTATION

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Since the discovery of the acceleration of alcoholic fermentation by thiamine (Schultz, Atkin & Frey, 1937*a*) and the development by these authors (see their review in Anderson, 1946) of their fermentation test, several attempts have been made to establish the nature of the effect. The problem is complicated by the fact that not all yeasts respond to thiamine in the same way, varying periods of incubation being required (e.g. Laser, 1941).

According to Sperber & Renval (1941) thiamine is taken up by yeast in two phases; a rapid absorption is followed by a slower conversion of absorbed thiamine into cocarboxylase. Westenbrink, Steyn-Parvé & Veldman (1947) also studied the conversion of thiamine into cocarboxylase by living yeast, but found that although 200–300 μg. of cocarboxylase could accumulate per gram of yeast, no increase in the enzyme carboxylase could be demonstrated.

Went (1949) on similar grounds, and also because the pyrimidine part of the thiamine molecule, which had been shown to accelerate fermentation (Schultz, Atkin & Frey, 1937*b*), apparently did not form cocarboxylase, suggested that the effect of thiamine on the fermentation rate did not involve the carboxylase system. An indirect effect in stimulating protein synthesis (in presence of ammonium salts) and increasing the rate of removal of 'high-energy phosphate bonds' was postulated, as this latter process was supposed to govern the rate of fermentation. Leijnse & Terpstra (1951), using a different technique of carboxylase estimation from that of Westenbrink *et al.* (1947), showed that the enzyme was formed on incubation of yeast for 4 hr., with either thiamine or 2-methyl-4-amino-5-ethoxymethylpyrimidine (hence the latter must have been converted into cocarboxylase), glucose and ammonium salts showing stimulatory actions.

In the previous paper (Trevelyan & Harrison, 1954) we have shown that, in fermentation by D.C.L. baker's yeast, a Michaelis-Menten equation may be used to describe the relation between fermentation rate and intracellular pyruvate level; and that one of the constants of this equation receives its simplest interpretation as the *in vivo* activity of the carboxylase system of the yeast cell. The present paper deals with the effect of thiamine, and of the pyrimidyl part of the thiamine molecule, on the fermentation rate-intracellular pyruvate relation. A diminution of pyruvate excreted by yeast into the medium, brought about by thiamine or cocarboxylase, has been reported by Haag & Dalphin (1940).

METHODS

Fermentation rate and pyruvate were determined as previously described (Trevelyan & Harrison, 1954). The yeast studied was again D.C.L. pressed baker's yeast. All experiments were carried out at 30°.

RESULTS

Effect of excess thiamine on pyruvate

These experiments were carried out using 20 or 30 ml. yeast suspension stirred by a stream of N_2, and contained in a 1 in. diameter Pyrex tube. In all cases 5% (w/v) yeast, and 0·1M glucose were used. Thiamine, when added, was 10^{-4}M, i.e. 2 μmoles/g. yeast. The effect of thiamine was to lower markedly both the extracellular and the intracellular pyruvate, as shown in Fig. 1. (It was immaterial whether the thiamine was added together with glucose at zero time, or had been previously added to the yeast, as in Fig. 1.) The effect was not dependent on the presence in the medium of ammonium salts, as Went (1949) claimed for the acceleration of fermentation rate, and low pyruvate values were obtained with yeast suspended in water (Fig. 2, A). When, after 30 min. fermentation in the presence of thiamine, the yeast was centrifuged, washed three times with water, and then used in a second fermentation, pyruvate values were still low (Fig. 2, B). In the experiment

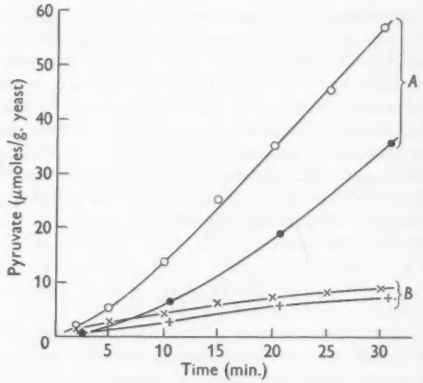

Fig. 1. Effect of thiamine (2 μmoles/g. yeast) on total and extracellular pyruvate in yeast fermentation. *A*, 5% (w/v) yeast; 0·1M glucose; 0·05M potassium succinate, pH 5·5; 0·005M-$(NH_4)_2HPO_4$. ○, total pyruvate; ●, extracellular pyruvate. *B*, as *A* with thiamine added. ×, total pyruvate; +, extracellular pyruvate.

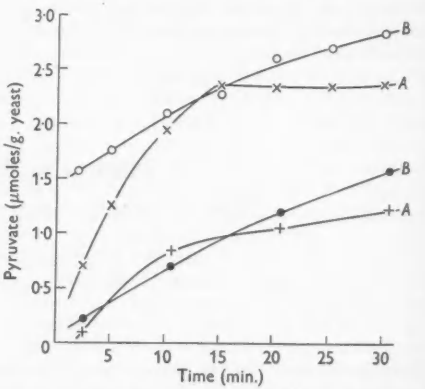

Fig. 2. Effect of thiamine (2 μmoles/g. yeast) on pyruvate during fermentation. *A*, unbuffered medium, pH 4·6. ×, total pyruvate; +, extracellular pyruvate. *B*, yeast previously allowed to ferment for 30 min. with thiamine and 0·1M glucose in potassium succinate, pH 5·5, then centrifuged and allowed to ferment in potassium succinate pH 5·5. ○, total pyruvate; ●, extracellular pyruvate.

of Fig. 3, the yeast was allowed to ferment for 32 min., to permit a high extracellular and intracellular pyruvate to be established, before the addition of thiamine (as 0·01 M solution). (Potassium succinate buffer was used as the medium, because pyruvate production between 10 and 30 min. had been found to be linear with time, hence the time course of pyruvate production before thiamine addition could be established using only two pyruvate determinations.) After addition of thiamine the production of extracellular pyruvate stopped, and a slight fall in pyruvate level set in. A dramatic fall in intracellular pyruvate followed thiamine addition, and was largely completed within 3 min. It is interesting to note that the highest extracellular pyruvate, at 35 min., was 1·0 μmole/ml. medium; the intracellular pyruvate fell to 1·8 μmoles/g. yeast, i.e. of the same order as the extracellular. Thus the effect of thiamine was clearly on the carboxylase system, either in (*a*) increasing the amount of carboxylase, (*b*) increasing the affinity of the enzyme for the substrate, or (*c*) increasing both. The rapidity of the effect made it unlikely that any increase in carboxylase could be due to synthesis of new protein. Cases (*a*) and (*b*) could in principle be distinguished by kinetic studies.

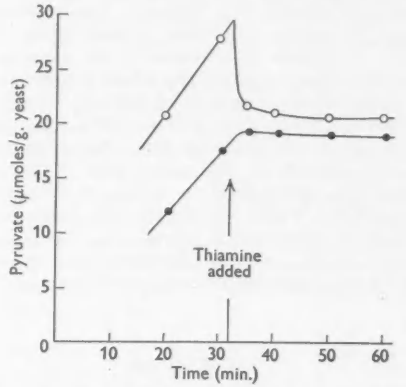

Fig. 3. Effect of thiamine (2 μmoles/g. yeast) added to established fermentation. 0·1 M glucose; potassium succinate buffer; pH 5·5. ○, total pyruvate; ●, extracellular pyruvate.

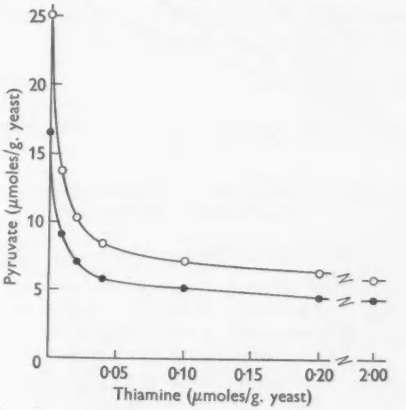

Fig. 4. Effect of amount of thiamine on total and extracellular pyruvate after 30 min. fermentation. 5% (w/v) yeast; 0·2 M glucose; sodium succinate buffer, pH 4·1; 0·01 M-KH_2PO_4; 0·01 M-$(NH_4)_2SO_4$; 0·0025 M-$MgSO_4$; 0·001 M-$ZnSO_4$; 0·001 M-$CaCl_2$; N_2 atmosphere. ○, total pyruvate; ●, extracellular pyruvate.

Effect on pyruvate of varying amounts of thiamine

The results are shown in Fig. 4. The medium used in the fermentations was that which gave the greatest pyruvate production in the absence of thiamine. An excess (2 μmoles/g. yeast) of thiamine caused a fall of 77% in total pyruvate; the yeast was, however, nearly saturated by 0·1 μmole/g. which caused a 72% drop, whilst as little as 0·01 μmole/g. gave a 45% fall. It is probable that a method for estimating thiamine could be based upon the pyruvate effect.

Kinetics of the thiamine effect

In order to decide between the two possible effects of thiamine on the yeast carboxylase system, attempts were made to determine the effect of thiamine on the two constants $V_{max.}$ and K_m of the Michaelis-Menten type relation connecting fermentation rate and intracellular pyruvate (cf. Trevelyan & Harrison, 1954). Fermentations with D.C.L. baker's yeast, under the conditions used in this work, take some 35 min. to reach maximum rate and steady intracellular pyruvate: at this time the determination by difference of intracellular pyruvate becomes uncertain, especially in the presence of thiamine, owing to the relatively high extracellular pyruvate. Attempts to use the increasing rate in maltose systems, as described in the previous paper (Trevelyan & Harrison, 1954), failed, because the thiamine effect, though rapid, was sufficiently time-dependent to make determinations at various times over 4 hr. not comparable. It had been noted that if, after 35 min. fermentation the yeast was centrifuged and washed, and used in a second fermentation, then the internal pyruvate reached its maximum value in a very short time. It was therefore decided to determine the effect of a preliminary fermentation with thiamine for a standard time. The determination of $V_{max.}$ and K_m depended upon only two points, one of which

was derived from an arsenate-inhibited system. The arsenate was not added in the first fermentation, so that the yeast absorbed thiamine under nearly the same conditions in all experiments. (The only difference was in the omission from the medium of phosphate, which counteracts the inhibitory action of arsenate.) The first fermentation was for 35 min. at 30° when glucose was the substrate, and for 90 min. with maltose, thiamine being added for the last 30 min. only. The yeast was then centrifuged, washed once with medium at 30°, and made up to 100 ml. with medium at 30°: 50 ml., containing 5 g. yeast, were immediately taken for the main fermentation. An unexpected advantage of this technique was that a tendency to foam in the xylene extraction step of the pyruvate estimation was eliminated, allowing double the amount of material to be used, with a consequent improvement in accuracy in estimating the low pyruvate level of arsenate-inhibited fermentation.

The following is a summary of the intracellular pyruvate results. They were determined at 5 min. intervals, starting at the time stated. *Expt.* 1, glucose fermentation, no thiamine: (5 min.) 12·0, 13·3, 15·0, 14·5. *Expt.* 2, glucose fermentation, arsenate, no thiamine: (10 min.) 1·15, 1·09, 1·02, 1·04. *Expt.* 3, glucose fermentation, 0·01 μmole thiamine/g. yeast: (5 min.) 3·9, 5·3, 5·9, 5·8. *Expt.* 4, glucose fermentation with arsenate, 0·01 μmole thiamine/g.: (10 min.) 0·78, 0·71, 0·69, 0·66. *Expt.* 5, maltose fermentation, 0·01 μmole thiamine/g.: (5 min.) 1·31, 1·30, 1·45, 1·46. *Expts.* 6 and 7 concerned the effect of higher thiamine concentrations, only the uninhibited glucose fermentation being studied. Pyruvate values at 10 and 20 min. were: *Expt.* 6, 0·02 μmole thiamine/g., 3·18, 2·74. *Expt.* 7, 0·10 μmole thiamine/g., 1·85, 1·39. Rate determinations were based on the CO_2 evolved over 10 min. intervals. In glucose fermentation they varied little after the first 10 min. The failure to observe the increasing fermentation rate in the presence of thiamine, described, e.g. by Schultz *et al.* (1937*a*, *b*), is due to the high yeast concentration used. In calculating the constants of the Michaelis-type equation the following points were used: for high-rate fermentations (Expts. 1, 3), mean pyruvate and fermentation rate at 10, 15 and 20 min., the rate at 15 min. being found by graphical interpolation; for arsenate fermentation (Expt. 4), mean pyruvate values at 15, 20 and 25 min. Mean values for the single maltose fermentation (Expt. 5) were used only as a check point.

Incubation with 0·01 μmole thiamine/g. does not alter the K_m value (Fig. 5): the values of 2·9 and 3·1 μmoles pyruvate/g. are the same as previously obtained (Trevelyan & Harrison, 1954). The maximum velocity $V_{max.}$, however, increases from 71 to 103 μmoles CO_2/min./g. From Expts. 3, 6 and 7 it appears that increasing the amount of thiamine does not affect the actual fermentation rate observed (medium as under Methods, Trevelyan & Harrison, 1954), hence in Fig. 5 all the thiamine points lie on a horizontal line. Assuming that $K_m = 3$ μmoles/g. in all cases, then 0·02 μmole thiamine/g. corresponds to a $V_{max.}$ of 135 μmoles CO_2/min./g., whilst saturating the yeast with 0·10 μmole thiamine/g. gives $V_{max.} = 192$. The change in $V_{max.}$ due to 0·02 μmole thiamine/g., viz. 64, is double that induced by 0·01 μmole/g.

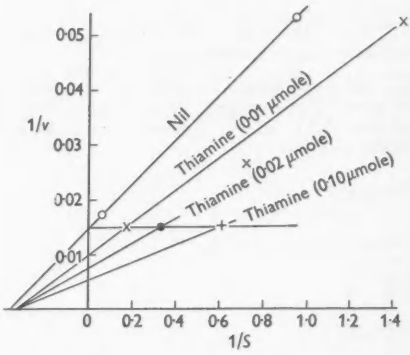

Fig. 5. Michaelis plot. v = fermentation rate; S = intracellular pyruvate.

Table 1. *Thiamine formation from 2-methyl-4-amino-5-ethoxymethylpyrimidine, calculated from pyruvate effect*

Time after adding pyrimidyl (min.)	Intracellular pyruvate S (μmoles/g.)	CO_2 rate v (μmoles/min./g.)	$V_{max.}$ $\left[=v\left(1+\frac{3}{S}\right)\right]$ (μmoles CO_2/min./g.)	$\Delta V_{max.}$* (μmoles CO_2/min./g.)	Thiamine* $\left(=\frac{\Delta V_{max.}}{3200}\right)$ (μmoles/g.)
5	11·3	58·2	74	3	0·0009
15	7·2	65·4	93	22	0·0069
25	5·9	66·0	100	29	0·0091
35	4·7	66·1	108	37	0·0115
45	3·9	64·6	114	43	0·0134

* Calculated by taking $V_{max.}$ as 71 μmoles CO_2/min./g., and $\Delta V_{max.}$ at a value of 32 μmoles CO_2/min./g. as equivalent to 0·01 μmole thiamine/g.

In Expt. 8, 0·02 μmole thiamine/g. was added only after fermentation with pretreated yeast had proceeded for 12 min. The pyruvate values at 10 min., i.e. before thiamine addition, were only 5% different from those of Expt. 1; 30 min. after thiamine addition the intracellular pyruvate was the same as in Expt. 6, where thiamine was present during the preliminary fermentation. The time course of the effect of 0·02 μmole thiamine/g. in Expt. 8 may be approximately translated into changes in $V_{max.}$ by applying the pyruvate values to the horizontal line of Fig. 5. Three minutes after thiamine addition $\Delta V_{max.} = 32$ μmoles CO_2/min./g.; after 8 min., 49; after 18 min., 52; after 30 min., 59.

Addition of the pyrimidyl part of the thiamine molecule (2 μmoles/g. yeast) also produced pyruvate changes, but the fall in pyruvate proceeded more slowly. The pyruvate changes may be transformed into $\Delta V_{max.}$ values, and using the relation that $\Delta V_{max.}$ of 32 μmoles CO_2/min./g. is equivalent to 0·01 μmole thiamine/g., the approximate amounts of thiamine apparently synthesized from 2-methyl-4-amino-5-ethoxymethylpyrimidine may be calculated; details of the calculation are summarized in Table 1.

DISCUSSION

The results support the view of Leijnse & Terpstra (1951) that thiamine, added during yeast fermentation, actually increases the amount of the enzyme carboxylase. The rapidity of its effect, however, suggests that with the yeast we have used it does so by supplying coenzyme to pre-existing apoenzyme, at least in the first 10 min. or so. Whether such formation of new carboxylase is reflected in an increased fermentation rate depends on carboxylase being the sole limiting enzyme (cf. Trevelyan & Harrison, 1954) for the yeast used. For the yeast studied here the fastest rate so far observed was 63 μmoles CO_2/min./g.; the $V_{max.}$ value for the carboxylase system was only 71, hence carboxylase activity could be said to limit fermentation rate. Thiamine, from 0·01 to 2·00 μmoles/g., gave a rate of 73; though $V_{max.}$ was increased up to about 200. This implies that another enzyme system now imposes a limit of 73 on the observed rate of CO_2 production. If amounts of thiamine over about 0·005 μmole/g. are to influence the fermentation rate, synthesis of non-carboxylase protein may well be required. It is obvious that different strains of yeast may react differently in regard to such factors as incubation time or presence of assimilable nitrogen.

We have seen that incubation of yeast for 30 min. with 0·01 μmole thiamine/g. increases $V_{max.}$ by 32 μmoles CO_2/min./g. Since the original $V_{max.}$ is 71, then the cocarboxylase content of the original yeast ought to be about 0·02 μmole/g. Actually the type of yeast used contains, by direct thiamine estimation, 0·01–0·02 μmole 'combined' thiamine per gram. Also, 1 mole cocarboxylase, according to these figures, catalyses the production of some 3000 moles CO_2/min. Jansen (1949) quotes a figure of 1500, based on the reconstitution of carboxylase in alkali-washed yeast. Green, Herbert & Subrahmanyan (1940, 1941) gave a value of 840 for their purified yeast enzyme.

There seems little doubt that the action of the pyrimidine part of the thiamine molecule depends upon its prior conversion into thiamine (cf. Leijnse & Terpstra, 1951). From Table 1 the rate of synthesis would appear to be some 3×10^{-4} μmole/min./g., which means that the thiamine content of the yeast would double after about 1 hr. Rates of a similar order for synthesis and degradation of coenzymes in various types of micro-organism have been reported by McIlwain (1947).

SUMMARY

1. Thiamine, added to fermenting baker's yeast, lowers the intracellular pyruvate corresponding to a given fermentation rate.

2. Kinetic studies suggest that this is caused, not by increased affinity of carboxylase for its substrate, but by the production of more enzyme.

3. The rapidity of action is interpreted as showing that, at least in the early stages, actual protein synthesis is not involved, i.e. that the process is one of supplying coenzyme to pre-existing apocarboxylase.

4. The pyruvate-lowering action of thiamine increases when 0·01–0·10 μmole/g. yeast is added, but the fermentation rate does not parallel thiamine increase above 0·01 μmole/g. It is pointed out that effects on CO_2 rate may depend upon the enzymic constitution of the particular yeast used.

5. The pyruvate-lowering action of 2-methyl-4-amino-5-ethoxymethylpyrimidine is explained as due to the synthesis from it of 3×10^{-4} μmole thiamine/min./g. yeast.

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The Estimation of Purines in Nucleic Acids: a Method Applicable to Materials with Low Concentration of Nucleic Acid

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During the course of studies on the metabolism of protein in the rumen of sheep, it became desirable to obtain an estimate of the content of nucleic acids in the digesta; this material consists of a heterogeneous mixture of plant material and a wide variety of bacteria and protozoa. No success was achieved in attempting to apply the procedures based on phosphorus partition (Schmidt & Thannhauser, 1945), colorimetric tests for pentoses (Schneider, 1945) or separation of the purines as the copper complexes (Graff & Maculla, 1935; Vendrely & Sarciron, 1944). Direct spectrophotometry, as used for example by Mitchell (1950), or chromatography, as developed by Markham & Smith (1949), could not be applied owing to the low concentration of nucleic acid in the materials analysed. No procedure has yet been devised for the quantitative separation of the nucleic acids from plant material. It was therefore decided to estimate the nucleic acids in terms of the purines, adenine and guanine; this was achieved by mild acid hydrolysis followed by precipitation of the purines as the silver salts (Schmidt & Levene, 1938; Gulland, Jordan & Threlfall, 1947), the separation of the two purines by chromatography (Markham & Smith, 1949) and final estimation by spectrophotometry. The method has been applied to preparations of protozoa and bacteria, plant leaves and other feeding stuffs as well as to digesta.

PROCEDURE

Preparation of sample. Before hydrolysis it is necessary to remove chloride and purine-containing compounds other than nucleic acids. Samples containing about 1 mg. of purine-N were extracted with 80% (v/v) ethanol containing approximately 0·001 N-H_2SO_4, in the case of dry samples, or treated with absolute ethanol to a final concentration of 80% in the case of aqueous suspensions.

The mixture was filtered with gentle suction over a thin layer of Celite, in a sintered glass funnel, and the precipitate was washed twice with 80% ethanol, once with absolute ethanol and once with acetone. The major part of the acetone was removed by suction and the last traces by drying the filter in an oven at low temperature.

Hydrolysis. The residue was hydrolysed in 17 ml. of N-H_2SO_4 in the boiling water bath for 1 hr. When handling coarse, fibrous plant material, considerable care was required at this stage. To prevent charring of polysaccharides the hydrolysis was performed in glass-stoppered tubes; the dry filter cake was thoroughly wetted with a little of the acid and transferred to the hydrolysis tube; the remainder of the acid was used to complete the quantitative transfer of the sample. At first, the mixture was cautiously warmed in the bath until occluded air was removed; the glass stopper was then inserted and the hydrolysis allowed to proceed.

In order to avoid errors due to change in volume of the acid during hydrolysis, the hydrolysate was separated from the bulky fibrous residue by straining through a disk of fine stainless steel gauze fitted in the bottom of an all-glass syringe; the fluid was expressed with the aid of compressed air and the pad compressed with the glass piston; the fibrous pad was washed in a similar manner several times with water. The completeness of washing could readily be verified by titration of residual acid in an aqueous extract of the fibre pad. The filtrate and washings were collected into a 25 ml. flask, made to volume and filtered through Whatman no. 42 paper.

Separation of the silver-purines. To 20 ml. of the filtrate was added 1 ml. of 0·5 M-$AgNO_3$ and the mixture was chilled in the refrigerator for 1–2 hr.; the inner wall of the centrifuge tube was then carefully rubbed down with a rubber-tipped glass rod to loosen any adherent precipitate. The rod was rinsed down with about 1 ml. of saturated Ag_2SO_4, in 0·01 N-H_2SO_4. After chilling again, the tubes were centrifuged at 0°, the supernatant fluid was removed and the wall of each tube carefully washed down with a small volume of the Ag_2SO_4 solution; this washing was best performed with a coarse Pasteur pipette carrying a short

length of rubber tubing (bicycle valve tubing) at the tip. The purpose of the washing is to ensure collection of the precipitate at the bottom of the tube rather than to remove contaminants. The tubes were again chilled and centrifuged; the supernatant was removed as completely as possible.

The precipitate was cautiously transferred with N-HCl, in small fractions, into a 1 ml. graduated pipette (previously recalibrated to contain known volumes of water) held vertically in a clamp; the transfer was controlled with a screw-plunger attached by pressure tubing to the pipette. This procedure was found to be simpler than transferring the precipitate to a 1 ml. volumetric flask. The contents were brought to the required volume and, after drying the tip of the pipette, were transferred to a small glass-stoppered tube; mixing was achieved by taking the fluid into and out of the pipette several times.

The preparation was allowed to stand for at least an hour with occasional mixing. The AgCl was then centrifuged down and the supernatant solution removed to a small glass-stoppered tube.

Chromatographic analysis. The purines were separated by the technique of Markham & Smith (1949, 1951) using Whatman no. 3 paper, and *n*-butanol:water:formic acid (77:13:10, v/v) in the descending direction. The purine spots were identified under ultraviolet light as described by Holiday & Johnson (1949). The spots and blanks were cut out in equal areas, extracted with 0·1N-HCl and the densities measured at wavelengths of 250 mμ. for guanine and 262 mμ. for adenine. For calculation, the molecular extinctions given by Smith & Markham (1950) and Wyatt (1951) were used.

Two procedures were found to be satisfactory for controlling the errors due to the paper blanks. In the first, triplicate portions of the sample were spotted on to the paper with alternating spots of solvent (N-HCl) for the blanks; in the second, multiple volumes (e.g. 0, 4, 8, 12, 16 μl.) were put on the paper and the final readings of density plotted on a graph to give a line the slope of which yields an estimate of the concentration corrected for blanks.

COMMENT ON PROCEDURE

Hydrolysis. Smith & Markham (1950) found that hydrolysis of ribonucleic acid with N-HCl at 100° for 1 hr. liberated the purine bases quantitatively; the pyrimidine bases remained in the form of nucleotides with traces of nucleosides. These latter compounds are not precipitated by Ag^+ in acid solution (Kerr & Seraidarian, 1945). Wyatt (1951) observed that the purines are rapidly liberated from deoxyribonucleic acids. Vendrely & Sarciron (1944) and Gulland *et al.* (1947) showed that these conditions of hydrolysis did not lead to a significant loss of purines by acid hydrolysis.

Paper blanks. Markham & Smith (1951) noted the importance of the paper blank as a contributing factor to analytical error, and overcame the difficulty by increasing the quantity of nucleic acid hydrolysate put on to the paper, and by using a chromatographic solvent containing strong hydrochloric acid. This tactic could not be used in the present work owing to the difficulty in obtaining the highly concentrated solutions required (equivalent to about 10 % solution of nucleic acid). The use of replicates or of multiple amounts proved to be satisfactory alternatives.

Solubility of silver-purines. The solubility of the silver salts of adenine and guanine in presence of excess Ag^+ was measured spectrophotometrically. For convenience the solvent chosen was Ag_2SO_4 (0·025N-Ag^+) in 0·6N-H_2SO_4. The solubility of Ag-guanine at 0° was found to be approximately $2{\cdot}3 \times 10^{-6}$M. The solubility of Ag-adenine was too low to be estimated by this means and hence may be taken as less than 1×10^{-7}M. In the procedures given here, these solubilities would indicate a source of error of about 0·5–1 % for guanine and less than 0·05 % for adenine.

Micro-pipettes. For delivery of small volumes of fluid on to the papers, a modification of the micro-pipette described by Wigglesworth (1937) has been used. The pipettes were made from short lengths of Pyrex capillary tubing with external diameter of about 5 mm. The tube was drawn out to a fine capillary, cut off and sealed into a suitable glass tube to act as a holder. The free end of the capillary was then drawn out, an approximate calibration made and the tube was finally cut at the required length. The pipettes were calibrated for delivery by weighing repeated deliveries of water on to a stack of filter paper disks in a small weighing bottle. The pipette is automatic in action, simple to use, accurate and sturdy. A similar pipette has been described by Meinhard & Hall (1950).

Sources of error. Two major sources of error have been noted. In work with gastric contents and with plant materials special care was required (as with all coarse heterogeneous materials) in taking the initial samples for analysis. The chief manipulative errors are associated with the paper chromatography. Provided that care is taken in the initial extraction of chloride and in removing the supernatant fluids from the Ag-purine precipitates, the volume occupied by the AgCl in the final extract produces a negligible error. In the course of 130 analyses of gastric contents, it was found that the deviation between duplicate samples averaged 2·6 %.

SUMMARY

A method is described for the estimation of the purines, adenine and guanine; it is based on the precipitation of the silver purines in acid solution followed by separation of the purines by paper chromatography and estimation by spectrophotometry. The method has been applied to yield an estimate of total nucleic acid in plant material and in the digesta of the ruminant stomach.

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The Oxidation of Myoglobin to Metmyoglobin by Oxygen

3. KINETIC STUDIES IN THE PRESENCE OF CARBON MONOXIDE, AND AT DIFFERENT HYDROGEN-ION CONCENTRATIONS WITH CONSIDERATIONS REGARDING THE STABILITY OF OXYMYOGLOBIN

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In Parts 1 and 2 (George & Stratmann, 1952*a*, *b*) it was shown that the oxidation of myoglobin to metmyoglobin by oxygen resembles very closely the corresponding oxidation of haemoglobin (Brooks, 1931, 1935). Only quantitative differences were found: in other respects the systems were identical. At constant oxygen pressure the oxidation rate is first order with respect to unoxidized myoglobin or haemoglobin; the rate constant at low oxygen pressures is proportional to the product of the concentrations of reduced myoglobin (Mb) and oxymyoglobin (MbO_2), or of reduced haemoglobin (Hb) and oxyhaemoglobin (HbO_2); at high oxygen pressures the rate constant is proportional either to $[MbO_2]$ or $[HbO_2]$, or to $[MbO_2]^2$ or $[HbO_2]^2$. Because results could not be obtained with sufficient precision at high oxygen pressures to decide between these alternatives, an attempt has now been made to decide between them by making use of the inhibition of myoglobin oxidation by carbon monoxide.

Brooks (1931) found that haemoglobin oxidation in air occurs more rapidly in more acid solutions. Similar experiments with myoglobin have now been carried out at both high and low oxygen pressures, over a range of hydrogen-ion concentrations.

Finally, some of the factors which contribute to the stability of oxymyoglobin and oxyhaemoglobin with respect to oxidation are discussed.

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MATERIALS AND METHODS

The myoglobin used was prepared as described by George & Stratmann (1952*a*). In the inhibition experiments, the myoglobin solutions were heavily buffered with K_2HPO_4 and KH_2PO_4, ratio 0·159/0·841, giving a total concentration of phosphate ions of 0·6 M with a pH of 5·69 at 30°. In the experiments in which pH was varied, the myoglobin was buffered with phosphate buffer mixtures prepared from NaH_2PO_4 and NaOH, giving pH values over the range 5·35–6·64. The ionic strengths of these buffer solutions were brought to 0·8, the same value as that of the 0·6 M potassium phosphate buffer, by the addition of appropriate quantities of NaCl. The pH of the myoglobin solutions was determined before each kinetic experiment using a glass electrode, calibrated with a 0·05 M potassium hydrogen phthalate buffer.

O_2, N_2, and CO were obtained from cylinders.

Kinetic measurements

The concentration of myoglobin was $1·68 \times 10^{-4}$ M. The reaction vessel, flow apparatus, and the procedure for analysis throughout the course of an experiment were as described by George & Stratmann (1952*a*, *b*). The results were recorded as the percentage of unoxidized myoglobin at various times from the start of the reaction. The log. of the percentage of unoxidized myoglobin was plotted against time in hours and a first-order rate constant, $k_{obs.}$ (hr.$^{-1}$), calculated from the slope of the line. In the experiments in which pH was varied, series of values for $k_{obs.}$ were obtained at two partial pressures of oxygen, 4 and 760 mm. In the inhibition experiments, CO–O_2 mixtures were used with partial pressures of CO(p_{CO}) between 0 and 45 mm. and of O_2(p_{O_2}) between 760 and 715 mm. All experiments were carried out at 30°.

RESULTS

Carbon monoxide inhibition

The oxidation of Mb to MetMb in mixtures of O_2 and CO of total pressure 760 mm., in which p_{CO} was varied from 10 to 45 mm. was found to be first order with respect to unoxidized myoglobin as is the case in the absence of carbon monoxide. This is illustrated in Fig. 1 where log. percentage (MbO_2 + MbCO) is plotted against time for experiments with p_{CO} 10 and 25 mm. Fig. 2 shows the relationships between the observed velocity constant $k_{obs.}$ (hr.$^{-1}$) and p_{CO}. The experiments at the highest values of p_{CO}, 35 and 45 mm., where half-reaction times were of the order of 7 hr., were less reproducible than the others, and the curve in Fig. 2 has been dotted in this region to indicate this uncertainty. The value of p'_{CO}, the partial pressure of carbon monoxide required to halve the uninhibited rate constant was 21 ± 2 mm.

These results can now be analysed to see what light they throw on the problem of which of the two alternative rate equations for the oxidation at high p_{O_2}, previously found to fit the experimental results equally well, is the correct one (George & Stratmann, 1952*b*),

$$-d[Fe_p^{2+}]/dt = 0{\cdot}30(1-\alpha)^2\,[Fe_p^{2+}], \qquad (1)$$

or

$$-d[Fe_p^{2+}]/dt = 0{\cdot}30(1-\alpha)\,[Fe_p^{2+}]. \qquad (2)$$

In these equations $[Fe_p^{2+}]$ stands for the total concentration of unoxidized myoglobin, i.e. [Mb] + $[MbO_2]$, and $(1-\alpha)$ gives the fraction present as MbO_2.

For the present experimental conditions, where the values of p_{O_2} and p_{CO} are such that all the myoglobin can be assumed to be present as MbO_2 and MbCO, it follows that

$$(1-\alpha) = 1\Big/\left\{\frac{Mp_{CO}}{p_{O_2}} + 1\right\},$$

where M, the partition constant given by

$$[MbCO]\,p_{O_2}/[MbO_2]\,p_{CO},$$

is left uncorrected for the different solubilities of O_2 and CO. Hence, according to equations 1 and 2 the rate constant is halved when either $(1-\alpha)^2$ or $(1-\alpha) = \frac{1}{2}$, corresponding to $Mp'_{CO}/p_{O_2} = 0{\cdot}41$ or 1. Using p'_{CO} 21 ± 2 mm. as determined above, and $p_{O_2} = 740$ mm. gives $M = 14{\cdot}5 \pm 1{\cdot}5$ or $M = 35 \pm 4$ respectively.

An attempt was made to determine M under identical experimental conditions by spectrophotometric estimation of MbO_2, MbCO and MetMb, in samples of myoglobin equilibrated in O_2–CO mixtures. Optical density measurements were made at 960 and 660 mμ. in the near infrared, where there are greater relative differences in absorption spectra than in the visible or Soret regions, and M was calculated by the method used by George & Stratmann (1952*b*) to obtain the oxygenation equilibrium constant. Very poor reproducibility was obtained, the mean of nine determinations giving 28 ± 8. It is in part attributable to the very large contribution to optical density from MetMb, unavoidably formed during equilibration, whose molar extinction coefficient at 960 mμ. was found to be 850, compared with 288 for MbO_2 and 5·3 for MbCO. With this method, any error in correcting for MetMb appears as a large error in M. It was therefore decided to rely on values in the literature, although these were obtained under different experimental conditions.

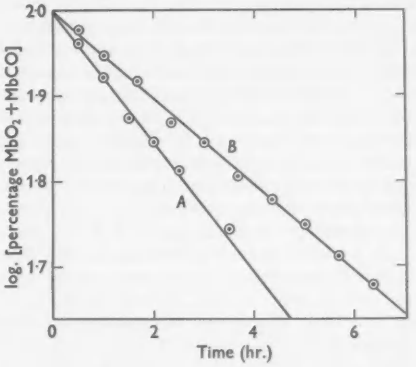

Fig. 1. First-order plots for the oxidation of myoglobin to metmyoglobin by oxygen at 30°, in 0·6 M phosphate buffer pH 5·7 in the presence of carbon monoxide. *A*, 10 mm. CO, 750 mm. O_2; *B*, 25 mm. CO, 735 mm. O_2.

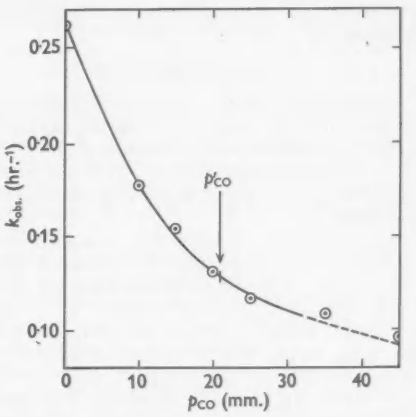

Fig. 2. The variation of the first-order rate constant, $k_{obs.}$ with the partial pressure of carbon monoxide, p_{CO}. The values for 35 and 45 mm. are less reliable than the others. The arrow indicates p'_{CO}, the partial pressure required to give a rate constant one half of the uninhibited rate constant.

Theorell (1934*a*) obtained values for M at pH 6·95 of $19·3 \pm 3·0$ at 20° and $13·8 \pm 2·8$ at 37°, from which the value 15·8 at 30° can be calculated using the van't Hoff isochore. This value has an error of about 18 %, i.e. $\pm 2·8$. It is unlikely that M would have an appreciably different value at the pH of 5·7 used in the present experiments as the following considerations show. First, the similarities between myoglobin and haemoglobin reactions extend to the way M varies with temperature; with haemoglobin the ratio of the values at 15 and 37° is 1·60 (Anson, Barcroft, Mirsky & Oinuma, 1925) compared with the value of 1·55 for myoglobin calculated from Theorell's data given above. Secondly, M for haemoglobin is pH independent, for although the equilibrium constants for HbO_2 and HbCO formation both show quite a marked pH dependence, it is of the same kind in both cases (Wyman, 1948). Some confirmation that the value of M is between 10 and 20 for myoglobin at 20° comes from Millikan's (1936) determinations of the velocity constants for the formation and dissociation of MbO_2 and MbCO at pH 7·4, namely $1·9 \times 10^7$ and 3×10^5 M^{-1} sec.$^{-1}$, and 37 and 0·04 sec.$^{-1}$ respectively. These constants are subject to an average error of 28 %, but as they stand they give $M = 14·6$. Using the temperature coefficient obtained from Theorell's data this becomes 12·0 at 30°.

There seems to be little doubt that under the present experimental conditions M would have a value somewhere between 13 and 19, and so equation 1, which gives $M = 14·5 \pm 1·5$, is to be preferred to equation 2, which gives $M = 35 \pm 4$, for the purpose of expressing the oxidation kinetics at high oxygen pressures.

Hydrogen-ion dependence

The first-order velocity constants, k_{760} and k_4, for the oxidation of myoglobin to metmyoglobin by oxygen in phosphate buffer solutions of pH 5·35 to 6·64, with ionic strength 0·80, $p_{O_2} = 760$ and 74 mm., are plotted in Fig. 3 against the hydrogen-ion concentration.

As shown above, equation 1 in which $k_{obs.} = 0·30\ (1-\alpha)^2$ hr.$^{-1}$, is to be preferred to equation 2 at high values of p_{O_2}. The corresponding equation at low values of p_{O_2} is $k_{obs.} = 2·62\alpha\ (1-\alpha)$ hr.$^{-1}$ (George & Stratmann, 1952*b*). Hence in analysing the variation of k_{760} and k_4 with $[H^+]$ it is first necessary to make allowance for the variation of $(1-\alpha)^2$ and $\alpha\ (1-\alpha)$ with $[H^+]$, since 760 and 4 mm. respectively fall in the ranges of p_{O_2} values where $k_{obs.}$ is given by these two expressions.

$(1-\alpha)$ and hence $(1-\alpha)^2$ is effectively constant at different pH values with $p_{O_2} = 760$ mm., since it is given by $p_{O_2}/(K_{O_2} + p_{O_2})$, where K_{O_2} is the dissociation constant of MbO_2, K_{O_2} having values of about 1 mm. for the present experimental conditions (Theorell, 1934*b*). Hence the variation of k_{760} with $[H^+]$ shown in Fig. 3 must arise through an ionization, or direct participation of hydrogen ions, at some stage in the actual oxidation mechanism.

Unlike $(1-\alpha)$, α is sensitive to changes in $[H^+]$ at all but extremely low values of $p_{O_2} < 0·05$ mm., because it is given by $K_{O_2}/(K_{O_2} + p_{O_2})$, and K_{O_2} shows a small but definite decrease as pH increases (Theorell, 1934*b*). log $(1/K_{O_2})$ plotted against pH is approximately linear, increasing by 0·098 and 0·116 per pH unit at 37 and 27° respectively. The difference is small, so rather than try to calculate a value for 30° based on ionization equilibria a mean value of 0·107 per pH unit has been taken, and from the value of $K_{O_2} = 1·13$ mm. at 30° and pH 5·7 obtained by George & Stratmann (1952*b*), the corresponding values of K_{O_2} have been calculated for each pH used in the present experiments. The true kinetic dependence on hydrogen-ion concentration of the velocity constants that go to make up k_4 can now be obtained by dividing k_4 by $K_{O_2} p_{O_2}/(K_{O_2} + p_{O_2})^2$, putting $p_{O_2} = 4$ mm. This new velocity constant will be called k_4'. The results of the calculations are summarized in Table 1, and the dotted line in Fig. 3 gives the plot of k_4' against $[H^+]$.

The plots in Fig. 3 show that with $p_{O_2} = 4$ and 760 mm. the variation of the rate constant is substantially less than the first-power variation, i.e. tenfold increase in K for a tenfold increase in $[H^+]$, which might result if the hydrogen ion were participating directly as a reactant at some stage in the oxidation process.

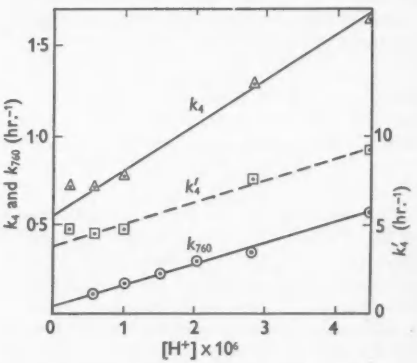

Fig. 3. The first-order velocity constants, k_4 and k_{760}, for the oxidation of myoglobin to metmyoglobin by oxygen in phosphate buffer solutions of pH 5·35 to 6·44 with ionic strength 0·8, at partial pressures of oxygen 4 and 760 mm. respectively, plotted against the hydrogen-ion concentration. The dotted line labelled k_4' is derived from the k_4 data by applying a correction described in the text.

Table 1. pH *and myoglobin oxidation*

Data for evaluating the hydrogen-ion dependence of the true oxidation velocity constant at low oxygen pressures, i.e k_4', using values of K_{O_2} calculated from a value of 1·13 mm. at pH 5·69 determined by George & Stratmann (1952*b*), and the variation of K_{O_2} with pH based on Theorell's measurements (1934*b*).

pH	k_4 (hr.$^{-1}$)	K_{O_2} (calc.) (mm.)	$\frac{4K_{O_2}}{(K_{O_2}+4)^2}$	$\frac{k_4(K_{O_2}+4)^2}{4K_{O_2}}=k_4'$
5·35	1·65	1·22	0·180	9·2
5·55	1·29	1·16	0·174	7·4
6·00	0·77	1·04	0·165	4·7
6·24	0·71	0·98	0·159	4·5
6·64	0·71	0·89	0·149	4·8

On the other hand, velocity constants in haemoprotein reactions often vary with $[H^+]$ because the ionization of one or more groups near the haem (or haemin) affect its reactivity. If K represents the ionization constant of one of these groups then its ionization can lead to a hydrogen-ion dependence of the form $[H^+]/(K+[H^+])$, $K/(K+[H^+])$, or some linear combination of the two, all of which can give a less than first-power variation with $[H^+]$. In haemoglobin compounds the pK values of such haem-linked ionizations are well established (Wyman & Ingalls, 1941), and recent calculations on data for myoglobin show it to have a linked group with a pK of about 6·6 in Mb and 6·3 in MbO_2 at temperatures of about 30° (George & Hanania, 1954). The data in Fig. 3 are not extensive enough to merit detailed mathematical analysis, but from the trend of the line it can be inferred that the variation of k_{760} with $[H^+]$ would follow if MbO_2 with the linked group in its conjugate acid form reacted several times more rapidly than the corresponding conjugate base. The variation of k_4' could be accounted for in a similar way, except that in this case both conjugate acid and conjugate base of Mb would have to be considered about equally reactive.

Brooks (1931) found that the velocity constant for haemoglobin oxidation in air also showed a less than first-order dependence on $[H^+]$, and it is very likely that this too could be explained as a consequence of haem-linked ionizations.

DISCUSSION

The rate equations previously proposed for the oxidation at low and high oxygen pressures are respectively:

$$\frac{-\mathrm{d}[Fe_p^{2+}]}{\mathrm{d}t}=\frac{k_a(1-\alpha)\,[Fe_p^{2+}]\,k_b\alpha[Fe_p^{2+}]}{k_c[Fe_p^{2+}]}, \qquad (3)$$

$$\frac{-\mathrm{d}[Fe_p^{2+}]}{\mathrm{d}t}=\frac{k_a(1-\alpha)\,[Fe_p^{2+}]k_b'(1-\alpha)\,[Fe_p^{2+}]}{k_c[Fe_p^{2+}]}, \qquad (4)$$

k_a is the velocity constant of a reaction of MbO_2 which initiates the oxidation, and k_b and k_b' are velocity constants of participating reactions of Mb and MbO_2 at low and high oxygen pressures respectively, all three of which are understood to be reactions of the haem iron atom. k_c is the velocity constant of another reaction in the overall process which apparently involves some other group in the molecule, because Mb and MbO_2 appear to undergo this reaction at equal rates. The inclusion of this reaction was essential to resolve the paradox that the rate at low pressures shows an oxygen pressure variation characteristic of the product

$$[Mb]\times[MbO_2], \text{ i.e. } \alpha(1-\alpha)\,[Fe_p^{2+}]^2,$$

whilst it is actually first order in $[Fe_p^{2+}]$.

In view of the first-order dependence on $[Fe_p^{2+}]$ at all oxygen pressures it is not surprising that the inhibited rate also shows the same behaviour (Fig. 1). The comparison of the partition constant M, obtained from the p_{CO} value necessary to halve the uninhibited rate constant, with the values obtained in gasometric experiments by Theorell (1934*a*) and from the velocity constants for O_2 and CO combination and dissociation (Millikan, 1936), supports a rate equation containing $(1-\alpha)^2$, as in equations 1 and 4, and not one containing $(1-\alpha)$, as in equation 2, to express the oxidation kinetics at high p_{O_2}. The first-order dependence on $[Fe_p^{2+}]$, and this quantitative check, support the general interpretation of the rate equations outlined above, for they imply that only the concentrations of the reactant species appearing in the numerator of the rate equation are diminished when carbon monoxide is present, and not that of the species in the denominator. In other words, the reaction with velocity constant k_c is unaffected when the haem iron atom combines with carbon monoxide just as it is apparently unaffected by the combination with oxygen.

The stability of oxymyoglobin with respect to its oxidation to metmyoglobin. Whatever the detailed mechanism of the oxidation, the fundamental electron transfer reaction (5)* will be among the

* In this chemical equation and those that follow, the symbol Fe_p^{2+} stands for reduced, i.e. uncombined, myoglobin, and not the unoxidized myoglobin as was the case in the previous kinetic equations 1 to 4.

most important governing the stability of oxymyoglobin,

$$Fe_p^{2+} + O_2 \rightarrow Fe_p^{3+} + O_2^-, \qquad (5)$$

for if this reaction is fast a rapid production of metmyoglobin will necessarily occur if it is not accompanied by significant back reactions regenerating myoglobin. Some indication of the speed can be inferred from an estimate of the heat of the reaction.

From a simple thermochemical cycle this can be shown to be $(E_{O_2} + S_{O_2^-}) - S_{O_2} - I_{Mb}$, where E_{O_2} is the electron affinity of the O_2 molecule, S_{O_2} and $S_{O_2^-}$ the solvation energies of the O_2 molecule and the O_2^- ion respectively, and I_{Mb} is the ionization potential of myoglobin in aqueous solution. Reasonable values of $(E_{O_2} + S_{O_2^-})$ and S_{O_2} are 79 and 3 kcal./g.mol. respectively and according to a very simple model, where only the change in charge on oxidizing Fe_b^{2+} to Fe_b^{3+} is considered, I_{Mb} would be about 90 kcal./g.mol. (George, 1952). Experimentally, I_{Mb} can be obtained from the heat of the cell reaction $Fe_p^{3+} + \frac{1}{2}H_2 \rightarrow Fe_p^{2+} + H^+$ which George & Hanania (unpublished work) calculated from preliminary results to be 89 ± 2 kcal./g.mol.; but an improved experimental technique now gives a higher value of 96 ± 2 kcal./g.mol. This must still be regarded as a provisional value because of experimental difficulties, but fortunately the uncertainty in no way affects the following argument. For if I_{Mb} is as high as 96 or as low as 89 kcal./g.mol., reaction (5) would be endothermic to the extent of 20 or 13 kcal./g.mol. respectively. Even if the reaction could occur with no additional activation energy, an abnormally high value of the temperature-independent factor A, in the Arrhenius equation $k = Ae^{-E/RT}$, of the order 10^{17} to 10^{21} l.mole^{-1} sec.$^{-1}$, compared with the usual value of about 10^{11} l.mole^{-1} sec.$^{-1}$, would be required to give k with a magnitude of 10^6 l.mole^{-1} sec.$^{-1}$ associated with 'fast' haemoprotein reactions.

The above calculations refer to the bimolecular reaction between myoglobin and oxygen resulting in electron transfer. For the fission of the complex itself with electron transfer as in reaction 6,

$$Fe_p^{2+}O_2 \rightarrow Fe_p^{3+} + O_2^- \qquad (6)$$

an additional factor, the exothermic formation of oxymyoglobin, must be considered. This enhances the resistance of myoglobin to oxidation by increasing the endothermicity of the overall electron transfer process as the following data show. The heat of formation of oxymyoglobin at the pH of 5·7 used by George & Stratmann (1952*b*) can be estimated as 19·5 kcal./g.mol. from Theorell's data (1934*b*). This becomes 16·4 kcal./g.mol. when corrected by 3·1 kcal./g.mol. for the heat of solution of oxygen, or 16 kcal./g.mol. to two significant figures. It therefore follows, by adding the heat of this reaction to that of 20 kcal./g.mol. calculated for reaction (5), that reaction (6) is endothermic to about 36 kcal./g.mol.

The detailed mechanism of these reactions may be envisaged with the help of the schematic potential energy diagram shown in Fig. 4. This diagram has been constructed from the thermochemical data and may be compared with that drawn up by Evans & Uri (1949) for their discussion of the formation of the $FeOH_{aq}^{2+}$ complex ion, from Fe_{aq}^{3+} and OH_{aq}^- ions, and the related reaction of Fe_{aq}^{2+} with the OH radical. The positions of state **A**, $Fe_p^{2+} + O_2$, and state **B**, $Fe_p^{2+}O_2$, are defined by the heat of formation of oxymyoglobin, and that of state **D**, $Fe_p^{3+} + O_2^-$, by the thermochemical cycle for reaction (5) above. State **C**, $Fe_p^{3+}O_2^-$, is placed 20 kcal./g.mol. above state **B** on the basis of the following consideration of the kinetic results given in George & Stratmann (1952*b*). The different form of the rate equations at low and high oxygen pressures, 4 and 760 mm., points to a common reaction in which Mb and MbO_2 respectively predominate, and in which oxidation of the iron occurs. The observed activation energies are 19 and 25 kcal./g.mol. respectively. The variation with temperature of the term involving K_{O_2} contributes an apparent activation energy of 14 kcal./g.mol. to that of the

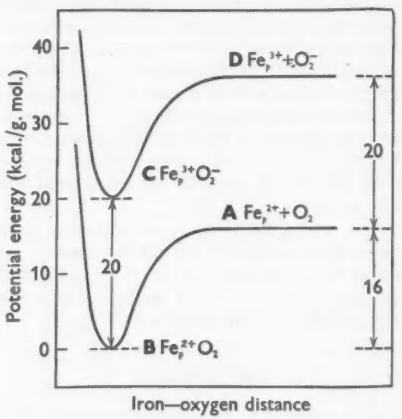

Fig. 4. Schematic potential energy diagram for the reaction of oxygen with myoglobin (Fe_p^{2+}) giving O_2^- and metmyoglobin (Fe_p^{3+}). In states **A** and **D**, Fe_p^{2+} and O_2, Fe_p^{3+} and O_2^- are infinitely separated. In state **B**, Fe_p^{2+} and O_2 have the internuclear separation characteristic of the stable oxymyoglobin molecule, $Fe^{2+}O_2$: state **C** is a hypothetical excited state, $Fe^{3+}O_2^-$, where electron transfer has occurred at this internuclear separation. States **A** and **B**, **C** and **D** are connected by lines drawn arbitrarily to give curves resembling the potential energy curves for diatomic molecules.

Mb reaction, so its real activation energy is 20 kcal./g.mol. less than that of the MbO_2 reaction. This difference of 20 kcal./g.mol. may be tentatively identified with the energy required to bring about the electron transfer $Fe_p^{2+}O_2 \rightarrow Fe_p^{3+}O_2^-$, since MbO_2 in this excited state could be expected to react as rapidly as Mb itself, where only electron transfer is required and no breaking of a Fe_p–O_2 covalent bond, in view of the high reactivity of the O_2^- in electron-transfer reactions.

Reaction 5 thus involves a change from state **A** to **B**, **B** to **C**, and finally from **C** to **D**, whereas reaction 6 only requires the change from **B** to **C**, and **C** to **D**. It is clear from Fig. 4, that because of the different initial state, reaction 5 is favoured to the extent of some 16 kcal./g.mol., provided that the exothermic heat of formation of $Fe_p^{2+}O_2$, the 16 kcal./g.mol., is not dissipated before electron transfer to state **C** has occurred. Summing up, in contrast to other classes of haemoproteins and haem derivatives, the exothermic formation of an oxygen complex by myoglobin and haemoglobin is itself a significant factor in protecting the haem from oxidation.

In view of these conclusions, reaction 5 is a more likely first stage of the oxidation than reaction 6 which was employed in the detailed mechanism discussed by George & Stratmann (1952*b*); but no distinction can be made between them on purely kinetic grounds. Consideration of the energetics places myoglobin in the same class as the ferrous ion regarding stability towards oxidation, for the ionization potential of ferrous ion in solution is 95 kcal./g.mol., compared with the provisional value of 96 kcal./g.mol. for myoglobin. Yet the oxidation of myoglobin is undoubtedly a faster reaction. Among many possible explanations, a reaction between MbO_2 and a reducing group present in the system could be considered as initiating the oxidation instead of reactions 5 or 6. Such an initiating reaction could easily be more rapid; but no scheme incorporating a reaction of this type has yet been found which would yield the observed kinetic behaviour. Nevertheless, the consumption of oxygen in excess of that required to oxidize the haem iron, described by George & Stratmann (1952*a*), does show that at some stage in the oxidation other groups in the molecule are attacked. This fact, together with the apparent stability of myoglobin according to the energetics, would provide a useful starting point for a future investigation.

SUMMARY

1. The oxidation of myoglobin (Mb) to metmyoglobin (MetMb) by oxygen is inhibited by carbon monoxide. The rate of oxidation in O_2–CO mixtures at atmospheric pressure, with partial pressure of carbon monoxide between 0 and 45 mm., pH 5·7 and 30°, is first order with respect to unoxidized myoglobin. The partial pressure of CO required to halve the uninhibited rate constant was 21 ± 2 mm.

2. The partition constant of myoglobin between CO and O_2 enters into the rate equations for the inhibited oxidation. Provided that a value of about 15 is appropriate to the present experimental conditions the results favour a rate equation containing $(1-\alpha)^2$ for the oxidation at high oxygen pressures, where $(1-\alpha)$ is the fraction of MbO_2.

3. In the pH range 5·35–6·64 at 30°, the rate of oxidation increases with hydrogen-ion concentration at both low and high oxygen pressures. The effect is small in magnitude, and could be due to the ionization of haem-linked groups on the myoglobin molecule.

4. A discussion of the available thermochemical data shows that electron transfer between Mb and O_2 giving MetMb and O_2^- is probably a relatively slow reaction since it would be endothermic to about 20 kcal./g.mol. The electron transfer dissociation of MbO_2 into MetMb and O_2^- would be much less favoured having an endothermicity of about 36 kcal./g.mol. on account of the large exothermic heat of formation of MbO_2. The formation of an oxygen complex by myoglobin and haemoglobin in an exothermic reaction is thus in itself a significant factor in protecting the haem from oxidation.

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South African Pilchard Oil

2. CONCENTRATES OF HIGHLY UNSATURATED FATTY ACIDS AND ALCOHOLS DERIVED FROM SOUTH AFRICAN PILCHARD OIL*

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The saponification of marine oils affords a complex mixture of fatty acids of widely differing chain length and degree of unsaturation. Acids of from 12 to 28 carbon atoms in chain length have been reported, the higher unsaturated members containing as many as six or seven double bonds per molecule (Lovern, 1942).

Analysis of such mixtures is greatly facilitated if preceded by a fractionation into groups of acids of largely similar properties. Preliminary segregation of a concentrate of highly unsaturated acids from the total hydrolysate has been achieved by several methods, among which are adsorption chromatography (Kaufmann, 1939, 1940), low temperature crystallization (Hilditch & Riley, 1945) and methods based on the relative solubility of the metallic salts of saturated and unsaturated acids in various solvents (Twitchell, 1921; Farnsteiner, 1903; Voorhies & Bauer, 1943). Of such solubility methods, the lithium salt–acetone procedure of Tsujimoto (1920) (cf. Tsujimoto & Kimura, 1923) has been claimed to be the most efficient in application to marine oils.

More recently, a new segregation procedure has been suggested by the fact that fatty acids of a high degree of unsaturation form complexes with urea less readily than do acids of lower unsaturation (cf. Schlenk & Holman, 1950). The efficiency of the urea complex method has been contrasted with that of the low temperature crystallization technique by Swern & Parker (1952), and in the present communication a comparison is made between the efficiency of the urea method and that of the lithium soap–acetone procedure for segregation of unsaturated acid concentrates.

As a basis for study, the lithium soap–acetone and urea complex procedures have been applied to the total acids from the body oil of the South African pilchard (*Sardina ocellata* Jenyns). Urea complex fractionation has also been applied to the unsaturated acid concentrate obtained by the lithium soap–acetone method, and the concentrates resulting from all procedures have been compared.

The alcohols obtained by lithium aluminium hydride reduction of pilchard glycerides have also been fractionated by the urea complex method. A concentrate of the more highly unsaturated alcohols has been compared with the corresponding unsaturated acids segregated by the urea and lithium soap–acetone procedures.

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EXPERIMENTAL

Unsaturated materials were sealed under high vacuum and stored at 0°. Wherever possible, operations with these materials were carried out under N_2.

Analytical methods

Iodine values were determined by the Wijs method as described in 'British Standards Specification (1950), Appendix E, p. 38', and a reaction time of 3 hr. was allowed.

Concentrates of unsaturated acids were completely hydrogenated over Adams's PtO_2 catalyst.

The molar percentages of the chain lengths present in the saturated material were determined by quantitative reversed-phase partition chromatography (Silk & Hahn, 1954).

Total pilchard fatty acids. Saponification of crude bleached pilchard oil gave the mixed fatty acids of mean equiv. wt. 282, and average number of double bonds/mol., 2·40 (H_2 uptake over Pd–$BaSO_4$).

Total pilchard fatty alcohols. A dry ethereal solution of pilchard oil (50 g.) was reduced by slow addition to an ethereal solution of $LiAlH_4$ (5 g.) (cf. Ligthelm, von Rudloff & Sutton, 1950). The total fatty alcohols (46 g., 99% yield) had an iodine value of 218.

Oxidation of pilchard alcohols to acids. To facilitate analysis, the concentrate of highly unsaturated alcohols was completely hydrogenated over Adams's PtO_2 catalyst and quantitatively oxidized to the corresponding mixture of acids with CrO_3 in glacial acetic acid (cf. Pollard, Chibnall & Piper, 1931).

Concentration of unsaturated acids by lithium soap–acetone procedure

Total pilchard acids (100 g.) in acetone (250 ml.) were neutralized at 15° with saturated aqueous LiOH solution (50 ml.). Acetone (700 ml.) was added to adjust the solvent concentration to 95%. The mixture was thoroughly shaken

and allowed to equilibrate for 4 hr. at 30° and then overnight at 15°. The precipitated lithium soaps were filtered off and discarded. The more highly unsaturated acids were recovered from the filtrate by acidification followed by extraction with *n*-pentane. The procedure was repeated once more with 95% aqueous acetone and finally with 97% aqueous acetone. Results are shown in Table 1.

Urea complex fractionation

The procedure was the same in general application to acids or alcohols.

Table 1. *Lithium soap–acetone segregation of pilchard unsaturated acids*

No. of treatments	Aqueous acetone (% acetone)	Weight (g.)	Equiv. wt.	No. of double* bonds/mol.
0	—	100	282	2·40
1	95	38	295	4·23
2	95	31	304	4·52
3	97	29·5	311	4·62

* H_2 uptake over Pd–$BaSO_4$.

General method. Total pilchard alcohols (45·4 g.) were dissolved in purified absolute ethanol (1200 ml.). Pure urea (50 g.) was added to the solution and dissolved by vigorous stirring and warming to 40°. After cooling slowly overnight, the crystalline adduct was filtered off and washed with cold ethanol (2 × 10 ml.).

Urea (20 g.) was added to the combined filtrate and washings and dissolved as before. After cooling overnight, the second crop of complexes was removed and washed. The process was repeated until the fourth crop of complexes had been obtained. The filtrates and washings were then evaporated to two-thirds of their volume under reduced pressure at 30°, and left to deposit the fifth crop on cooling. Thereafter the solution was concentrated somewhat at each step so that 5–10 g. of urea complexes were obtained on cooling. The mother liquors from the final precipitation were evaporated to yield a concentrate of the more highly unsaturated alcohols. The complexes precipitated at each stage were decomposed with water and the fatty material was recovered by ether extraction.

The method could be adjusted to afford a greater or lesser number of fractions as required, while by exhaustive precipitation with urea in the case of the alcohols, it was possible

Table 2. *Urea complex fractionation of total pilchard fatty acids*

Fraction	Weight (g.)	Weight (As % of starting material)	Equiv. wt.	No. of double* bonds/mol.	Iodine value
Total fatty acids	95·0	100·0	282	2·40	201
1	5·0	5·6	278	0·20	12·0
2	13·5	15·0	276	0·35	20·7
3	13·5	15·0	271	0·46	46·7
4	17·0	19·0	280	1·49	135
5	12·0	13·3	298	3·65	300
6	2·0	2·2	305	4·54	—
7	0·5	0·6	310	—	—
8	4·0	4·5	304	4·55	368
9	1·0	1·1	302	—	368
10	1·0	1·1	305	4·65	—
Non-complex forming residue	20·0	22·0	304	4·89	385·7

* H_2 uptake over Adams's catalyst.

Table 3. *Urea complex fractionation of unsaturated acids segregated by the three-stage lithium soap–acetone procedure*

Fraction	Weight (g.)	Weight (As % of starting material)	Equiv. wt.	No. of double* bonds/mol.
Li soap–acetone concentrate	93·0	100·0	307	4·55
1	4·8	5·16	315	3·00
2	2·48	2·67	304	3·38
3	1·85	1·99	300	3·39
4	4·40	4·74	300	4·01
5	2·58	2·78	302	4·21
6	1·50	1·61	304	4·40
7	3·95	4·25	304	4·54
8	1·90	2·04	303	4·60
9	4·05	4·36	301	4·69
10	2·24	2·41	305	4·82
11	0·82	0·88	303	4·71
Non-complex forming residue	55·0	59·1	308	4·85

* H_2 uptake over Pd–$BaSO_4$.

to obtain a non-complex forming fraction representing approximately 12% of the total.

Total acids. The urea complex fractionation of total pilchard fatty acids is illustrated in Table 2.

Lithium soap–acetone concentrate of unsaturated acids. Further subdivision by means of the urea method of the lithium soap–acetone concentrate prepared as described in Table 1 is illustrated in Table 3.

Total alcohols. The urea complex fractionation of total pilchard alcohols is illustrated in Table 4.

Chain length analysis and properties of the concentrates obtained by the lithium soap–acetone and urea complex fractionation procedures

Concentrates were quantitatively chromatographed after saturation, the alcohols being converted into the corresponding acids. The results are summarized in Table 5.

DISCUSSION

Four concentrates of highly unsaturated material have been prepared from total pilchard fatty acids and alcohols.

Table 4. *Urea complex fractionation of total pilchard fatty alcohols*

Fraction	Weight (g.)	Weight (As % of starting material)	Iodine value
Total fatty alcohols	45·4	100·0	218
1	3·10	7·5	16·6
2	6·75	16·3	25·5
3	3·30	8·0	52·2
4	4·10	9·9	96·9
5	2·00	4·8	147
6	4·35	10·5	228
7	1·60	3·9	297
8	1·80	4·3	344
9	0·95	2·3	358
10	1·05	2·5	379
Non-complex forming residue	12·45	30·0	365

(*A*) A concentrate of highly unsaturated acids prepared by application of the lithium soap–acetone procedure to total pilchard fatty acids.

(*B*) A concentrate of highly unsaturated acids prepared by application of urea complex fractionation to total pilchard fatty acids.

(*C*) A concentrate of unsaturated acids prepared by urea complex fractionation of concentrate *A*.

(*D*) A concentrate of unsaturated alcohols prepared by urea complex fractionation of total pilchard fatty alcohols.

Direct comparison of concentrates *A* and *B* shows that the latter is of greater average unsaturation and, moreover, contains proportionately less of the longer-chain C_{20} and C_{22} acids. It is thus apparent that the urea procedure is more efficient than the lithium soap for removal of long-chain acids of low unsaturation. For the purpose of preparing a concentrate rich in shorter-chain highly unsaturated acids, the urea method is therefore to be preferred. The better stepwise fractionation obtainable with the urea method would also commend its use in preference to the lithium soap procedure for this purpose.

Comparison of concentrates *B* and *C* shows that both have the same average unsaturation, but that the latter is proportionately richer in the longer-chain C_{20} and C_{22} unsaturated acids. Thus if a preliminary lithium soap–acetone segregation is carried out before application of the urea method, then the final unsaturated concentrate is richer in C_{20} and C_{22} unsaturated acids than the concentrate obtained by direct application of the urea method to total pilchard acids. The lithium soap–acetone procedure is thus effective in removing an appreciable quantity of C_{16} and C_{18} acids of relatively low unsaturation which are not easily removed by the urea method. This is consistent with the first observation that the lithium soap–acetone procedure, in direct application to total pilchard acids, affords a concentrate

Table 5. *Comparison of concentrates after saturation*

Material	Yield (as % of total)	Equiv. wt. (unsaturated)	No. of double bonds/mol.	Molar % C_{16}	Molar % C_{18}	Molar % C_{20}	Molar % C_{22}
Total pilchard fatty acids	100	282	2·40*	18·25	23·9	39·6	18·25
A. Li soap–acetone segregated acids	32	307	4·55*	23·5	12·0	40·8	23·6
B. Urea complex segregated acids	22	304	4·88†	29·6	18·6	31·9	19·9
C. Urea complex segregated acids ex Li soap–acetone concentrate	19·7	308	4·85*	19·9	13·4	38·6	28·1
D. Urea complex segregated alcohols	37·6	—	‡	29·0	11·9	43·5	15·6

* H_2 uptake over Pd–$BaSO_4$.
† H_2 uptake over Adams's catalyst: corresponding iodine value, 386.
‡ Iodine value, 371.

richer in longer chain acids than does the urea method.

Application of the urea procedure to total pilchard fatty alcohols affords a satisfactory stepwise fractionation, resulting in a concentrate rich in C_{16} and C_{20} unsaturated alcohols with a relatively small proportion of the C_{18} and C_{22} chain lengths.

Each of the four procedures investigated would appear to have its respective merits depending upon the nature of the unsaturated concentrate required. Material relatively rich in any desired chain length can be prepared for subsequent isolation of individual acids or alcohols by choice of the appropriate procedure. For convenience in analysis, fractionations involving the acids rather than the alcohols are to be preferred.

Urea fractionation of acid esters was not undertaken, although this has been claimed by Abu Nasr & Holman (1951–2) to result in the removal of material more highly unsaturated than is the case when the free acids are fractionated. The reduced tendency of the esters to associate is offered as an explanation of this behaviour.

SUMMARY

1. The urea complex fractionation and lithium soap–acetone techniques have been applied to the segregation of a concentrate of highly unsaturated fatty acids from the body oil of the South African pilchard. For comparison, the alcohols resulting from lithium aluminium hydride reduction of pilchard glycerides have also been fractionated by the urea complex method.

2. A comparison has been made between the unsaturated concentrates prepared by each procedure. The urea complex method affords an excellent stepwise fractionation resulting in a concentrate of high average unsaturation rich in the shorter C_{16} and C_{18} chain lengths. The lithium soap–acetone procedure affords a concentrate of lower average unsaturation than does the urea method, but the segregate is relatively richer in the longer C_{20} and C_{22} chain lengths. Urea fractionation of total pilchard fatty alcohols affords a concentrate of high average unsaturation rich in the C_{16} and C_{20} chain lengths.

The authors wish to thank Dr D. A. Sutton and Dr W. S. Rapson, Director of the National Chemical Research Laboratory, for their interest and advice throughout the undertaking of this work. Part of the investigation was made possible through the endowment of a Fellowship by Messrs Marine Oil Refiners of Africa, Ltd., Simonstown, Cape Province, which is gratefully acknowledged. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

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South African Pilchard Oil

3. THE FATTY ACID COMPOSITION OF SOUTH AFRICAN PILCHARD OIL

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The complete and unambiguous analysis of the fatty acid composition of marine oils is a matter of extreme complexity owing to the diverse nature of the component acids. Not only are acids of differing chain length and unsaturation encountered, but also acids which have the same chain length and a varying degree of unsaturation. Acids of from 12 to 28 carbon atoms in chain length have been reported; the higher members containing as many as six or seven double bonds per molecule (Lovern, 1942).

A large number of the more highly unsaturated acids of marine oils have been isolated and character-

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Table 1. *Molecular distillation of the unsaturated acid concentrate*

Fraction	Distillation temperature (°)	Weight (g.)	Equiv. wt.	Refractive index (n^{30})	No. of double* bonds/mol.
Unsaturated acids	—	29·5	311	—	4·62
I	96	3·40	271	1·4806	3·55
Intermediate	105	0·72	274	1·4832	3·62
II	105	2·84	276	1·4848	3·65
Intermediate	120	0·59	287	1·4879	4·08
III	120	2·51	287	1·4886	4·18
Intermediate	130	0·47	306	1·4913	4·65
IV	130	4·83	307	1·4933	4·86
V	135	3·57	315	1·4950	5·20
VI	145	2·27	327	1·4972	5·42

Residue: 7·0 g. Hold-up and loss: 1·3 g.
* H_2 uptake over Pd–$BaSO_4$.

ized, chiefly by the Japanese workers (e.g. Toyama & Tsuchiya, 1935*a*, *b*; Tsujimoto, 1906; Toyama & Yamamoto, 1953; Ueno & Iwai, 1934; Ueno & Yonese, 1936). While such work has been of very great value, the results are nevertheless open to some criticism on the grounds that the more labile components could easily have been modified during the vacuum distillation used for their isolation. The prolonged heating of acids with more than three double bonds per molecule is known to result in isomerization and polymerization reactions (cf. Norris, Rusoff, Miller & Burr, 1941, 1943).

For analysis of fatty acid mixtures without isolation of individual components, the well established method of Hilditch has been almost universally employed. With all its merits, this method does nevertheless suffer from a disadvantage in regard to thermal modification of the components, and is moreover only empiric in regard to the assignment of unsaturation between the different chain lengths in the case of marine oil acids.

The present communication represents a new approach to the analysis of marine oils without isolation of individual component acids. The techniques of lithium soap–acetone segregation, molecular distillation, reversed-phase partition chromatography and urea complex fractionation have been employed in successive subdivision of pilchard acid fractions. By suitable application of these techniques it has proved possible to obtain a fraction whose composition could be calculated unambiguously without isolation of an individual component. The procedures employed can reasonably be expected to have caused little or no modification of the original acids.

EXPERIMENTAL

General methods. The preparation of the total pilchard fatty acids and the concentration of the unsaturated fatty acids were as described in the preceding paper (Silk, Sephton & Hahn, 1954).

The concentrate of the more highly unsaturated acids prepared using the lithium soap–acetone technique represented 29·5% of the total acids; it had an average equiv.wt. of 311, and an average number of double bonds/molecule, 4·62 (H_2 uptake over Pd–$BaSO_4$).

Table 2. *Chain length distribution of fractions I–VI*

Fraction	Molar percentages C_{16}	C_{18}	C_{20}	C_{22}
I	62·8	13·2	17·0	7·0
II	41·3	18·2	32·1	8·4
III	19·7	20·8	49·2	10·3
IV	5·1	10·3	64·2	20·4
V	1·9	4·4	64·5	29·2
VI	2·0	2·6	44·9	50·5

Molecular distillation of the unsaturated acid concentrate

A three-stage falling-film molecular still, especially designed for the purpose (Sutton, 1953), was used to separate the unsaturated acid concentrate into six fractions (I–VI) of increasing boiling point and equivalent weight. The distilland was recycled twice with all distilling stages at the same temperature before removal of each fraction, and the temperature of the stages was increased by use of a higher boiling solvent after each successive fraction had been removed.

Results are shown in Table 1.

Reversed-phase partition chromatography of fractions from the molecular distillation process

The six fractions (I–VI) from the molecular distillation process were chromatographed (23 mg. each) on a column 31·5 cm. in height × 1·4 cm. diameter packed with non-wetting kieselguhr supporting liquid paraffin as the stationary phase (cf. Silk & Hahn, 1954*a*). Aqueous acetone (60%, v/v) was used to develop the chromatograms at a flow rate of 35 ml./hr. and a temperature of 10°. The curves obtained are shown in Fig. 1.

Chain length analysis of the six unsaturated acid fractions (I–VI) from the molecular distillation process

Each of the fractions was completely hydrogenated over Pd–$BaSO_4$ catalyst, and the resulting mixture of saturated acids analysed for chain length distribution by reversed-phase partition chromatography (Silk & Hahn, 1954*a*). Results are given in Table 2.

Table 3. *Urea complex fractionation of fraction I*

Fraction	Weight (g.)	Equiv. wt.	No. of double bonds/mol.	Molar percentages C_{16}	C_{18}	C_{20}	C_{22}
I (total)	2·02	271	3·55	62·8	13·2	17·0	7·0
Iα	0·14	281	1·66	69·0	19·0	10·5	1·5
Iβ	0·18	273	2·55	59·5	13·8	22·6	4·1
Iγ	0·08	276	3·14	—	—	—	—
Iδ	0·04	275	3·34	—	—	—	—
Residue	1·5	263	3·68	79·3	11·0	9·7	0

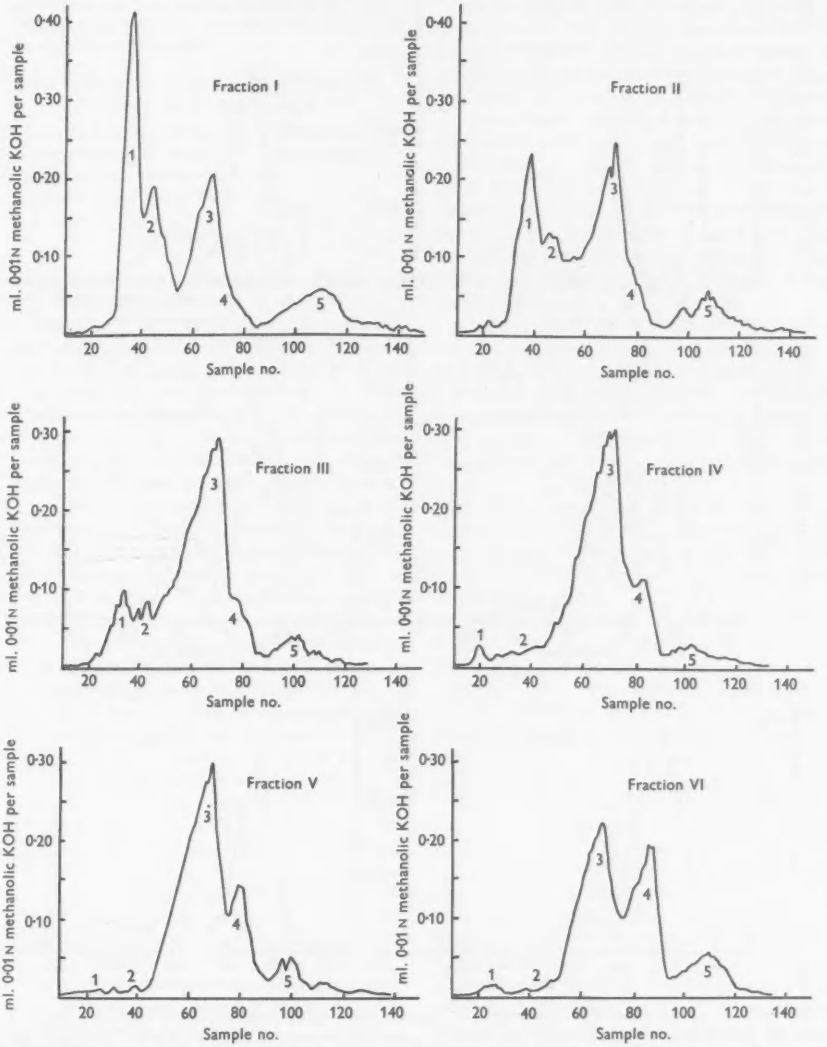

Fig. 1. Reversed-phase partition chromatography of unsaturated acid fractions I–VI from molecular distillation. Solvent system: aqueous acetone (60%, v/v)–medicinal paraffin supported on non-wetting kieselguhr. Column: 1·4 × 30·0 cm. Temp. 10°. Flow rate: 35 ml./hr. Samples 1·4 ml. Load: 23 mg. each fraction.

Urea complex fractionation of fraction I from the molecular distillation

Fraction I was further fractionated by removal of the components of lower unsaturation as their complexes with urea. Thus, urea (3 g.) was dissolved in absolute ethanol (80 ml.) and the solution cooled to 15°. Fraction I (2·02 g.) was added to the solution and the whole left overnight at 4°. The crystalline precipitate was filtered off and washed with cold ethanol. The filtrate and washings were concentrated to a volume of 65 ml. and left to deposit a second crop of complexes which were removed by filtration. Urea (0·5 g.)

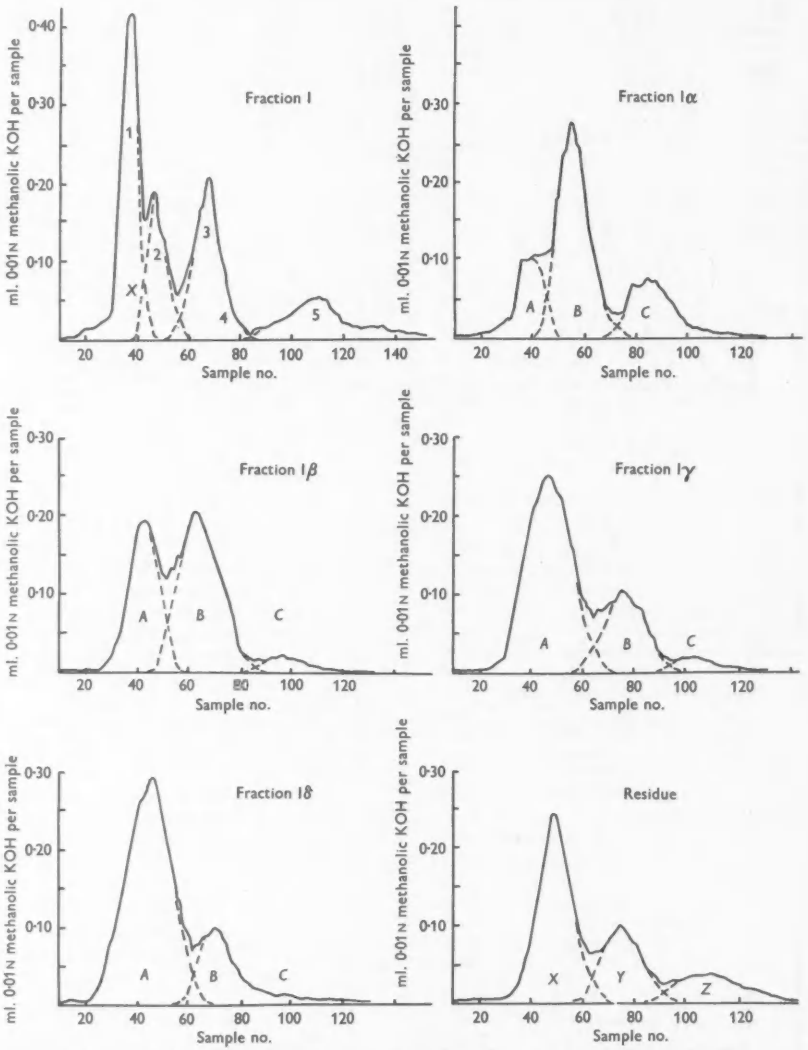

Fig. 2. Reversed-phase partition chromatography of unsaturated acids segregated from fraction I by urea complex fractionation. Stationary phase: medicinal paraffin supported on non-wetting kieselguhr. Moving phase: aqueous acetone. Fraction I, 60%, v/v; fraction Iα, 70%, v/v; fraction Iβ, 67·5%, v/v; fractions Iγ and Iδ, 65%, v/v; residue, 55%, v/v. Column: 1·3 × 35 cm. Temp. 10°. Flow rate: 35 ml./hr. Samples: fraction I, 1·4 ml.; fractions Iα–Iδ and residue, 1·0 ml. (Residue curve: molar percentage peak *X*, 52·7%; peak *Y*, 26·6%; peak *Z*, 20·6%.

was added to the filtrate and washings, which were allowed to deposit a third crop of complexes on standing. A fourth crop was obtained by evaporation of the filtrate from the previous crop to a volume of 30 ml. and again allowing to stand overnight at 4°. The acids which did not form urea complexes were recovered from the final filtrate by evaporating it to dryness, adding water and extracting with *n*-pentane. The complexes precipitated at each stage in the fractionation were decomposed with water, and the acids recovered by extraction with *n*-pentane.

The subdivision of fraction I is illustrated in Table 3. Chain length analyses of fractions removed as their urea complexes are shown.

Reversed-phase partition chromatography of subfractions I α–I δ and residue

The subfractions Iα–Iδ and the residue obtained by urea complex fractionation of fraction I were subjected to reversed-phase partition chromatography.

The results are illustrated in Fig. 2.

DISCUSSION

Inspection of the chromatographic curves for fractions I–VI shows that as peaks 1 and 2 on the left-hand side diminish in quantity, so peak 4 on the right-hand side increases, while peak 3 reaches a maximum and then again diminishes. This behaviour is paralleled by the variation in chain length distribution of fractions I–VI (Table 2), from which it is seen that as the molar percentage of lower boiling C_{16} acids shows a steady decrease, so the percentage of higher boiling C_{22} acids shows an increase, while the percentage of $C_{18}+C_{20}$ acids reaches a maximum and then decreases. A broad assignment of chain length to the peaks shown in the chromatographic curves is thus possible. Peaks 1 and 2 may well represent C_{16} acids, peak 4 C_{22} acids, peak 3 a mixture of $C_{18}+C_{20}$ acids, and peak 5 a complex mixture of variable composition probably consisting largely of C_{22} acid in fraction VI.

Such correlation of chain length with peaks in a chromatographic curve is of no value unless the effect of unsaturation upon the resolution is taken into account. In our experience, the clear separation shown on chromatography of a mixture of oleic and linoleic acids has suggested that acids of the same chain length but different degree of unsaturation should be easily resolvable. The failure to resolve a mixture of palmitic and oleic acids has suggested that where one component has a double bond, and two methylene groups more than the other, resolution is not possible. These findings have recently been confirmed by Savary & Desnuelle (1953), and in their studies on the countercurrent distribution of fatty acids Ahrens & Craig (1952) have arrived at similar conclusions.

Further subdivision of one of the crude molecular distillates (fraction I) has been achieved by means of urea complex fractionation. Chromatography of the urea segregates (fractions Iα–Iδ) shows curves (Fig. 2) in which a well defined variation of the peaks is apparent. Peak *A* increases throughout the series, while *B* and *C* diminish. These fractions are nevertheless still too complex to permit unambiguous calculation of their composition.

The non-complex forming residue obtained by urea fractionation of fraction I is, on the other hand, a relatively simple mixture of acids whose composition can be calculated without isolation of individual components. The chromatographic curve of this unsaturated material shows three peaks (Fig. 2) whose molar percentages are 52·7, 26·6 and 20·6% respectively. The chain length analysis of this fraction (Table 3) shows that no C_{22} acid is present. The molar percentage of C_{16} acid (79·3%) suggests that peaks *X* and *Y* in the unsaturated curve (Fig. 2) are both C_{16} acid, since the sum of their molar percentages (52·7 + 26·6) is in agreement with the C_{16} value determined. Peak *Z* must therefore represent a mixture of C_{18} and C_{20} acids, in the molar proportions of 11·0 and 9·7 respectively (cf. Table 3).

In the assignment of unsaturation to the peaks of the residue curve (Fig. 2), it is reasonable to assume that peak *X* is a C_{16} tetraene acid and peak *Y* a C_{16} triene, since resolution might be expected between such a pair of acids with the more highly unsaturated member being eluted first. The isolation and characterization of a C_{16} tetraene acid are reported in the succeeding paper (Silk & Hahn, 1954*b*). Peak *Z* of the residue curve (Fig. 2) is likely to be a mixture of C_{18} triene and C_{20} tetraene acids, since these would be expected to give an overlapping peak and yet both be resolvable from C_{16} tetraene and triene acids.

Assuming therefore that the residue curve (Fig. 2) represents a mixture of 52·7% C_{16} tetraene, 26·6% C_{16} triene, 11% C_{18} triene and 9·7% C_{20} tetraene, then the average equivalent weight calculated for such a mixture would be 259 and the average number of double bonds per molecule = 3·62. These values are in excellent agreement with the values of 263 and 3·68 experimentally determined (Table 3), and indeed it is not possible to postulate any other composition for the residue curve such that the theoretically calculated values for equivalent weight and average unsaturation agree with those experimentally observed.

Analysis of the residue curve (Fig. 2) indicates that by successive subdivision of pilchard acid fractions it is possible to obtain a mixture of a sufficiently simple nature to permit calculation of its composition without isolation of individual components. Other fractions obtained were still too complex in nature to allow of more than qualitative analysis, but it may be hoped that further subdivision of these mixtures by the techniques

suggested will result in less-complex fractions amenable to quantitative analysis. The mild procedures employed in fractionation of the acids might be expected not to cause modification of structure during processing. Consideration of the results obtained in this investigation illustrates that the composition of marine oils is of a far greater complexity than hitherto envisaged.

SUMMARY

1. A new approach has been suggested for the analysis of total marine oil acids without isolation of individual components.

2. The techniques of lithium soap–acetone segregation, molecular distillation, reversed-phase partition chromatography and urea complex fractionation have been applied in the successive subdivision of pilchard acid fractions. Relatively simple mixtures of acids have been obtained; the composition of one of which has been calculated without isolation of an individual component.

3. The techniques employed have been such as might be expected to have caused little or no modification of the component acids.

The authors wish to thank Dr D. A. Sutton for his interest and advice throughout the undertaking of this work which was made possible through the endowment of a Fellowship by Messrs Marine Oil Refiners of Africa Ltd., Simonstown, Cape Province. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

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South African Pilchard Oil

4. THE ISOLATION AND STRUCTURE OF A HEXADECATETRAENOIC ACID FROM SOUTH AFRICAN PILCHARD OIL

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In the preceding communication of this series (Silk & Hahn, 1954), the presence of a C_{16} tetraene acid in the body oil of the South African pilchard (*Sardina ocellata* Jenyns) was posulated in order to account for certain observed analytical values. The isolation of such an acid is now reported, together with its characterization by physical and chemical means.

The techniques of lithium soap–acetone segregation, urea complex fractionation and molecular distillation have been applied in successive subdivision of total pilchard acids yielding a concentrate of highly unsaturated material. A C_{16} tetraene acid has been isolated from this concentrate by reversed-phase partition chromatography on a preparative scale, and has been characterized as *hexadeca*-6:9:12:15-*tetraenoic acid*. The techniques employed for its isolation have been sufficiently mild in nature to render it unlikely that any modification of structure could have taken place during processing.

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† Assistant in the Fellowship.

EXPERIMENTAL

General methods. See Silk, Sephton & Hahn (1954).

Ultraviolet spectra were determined on a Beckman model DU quartz spectrophotometer. Infrared spectra were determined on a Perkin-Elmer single-beam infrared spectrophotometer.

Table 1. *Molecular distillation of the concentrate of unsaturated acids*

Fraction	Distillation temp. (°)	Weight (g.)	Equiv. wt.	Refractive index (n_D^{31})	No. of double bonds/mol.
Concentrate of unsaturated acids	—	54·3	308	—	4·85
A	85	2·60	260	1·4801	3·67
B	90·5	1·78	263	1·4811	3·72
C	96	5·53	268	1·4827	3·93
Residue *R* (3rd stage)	—	1·18	295	1·4889	4·54
Main bulk residue	—	approx. 41·7	325	—	5·38

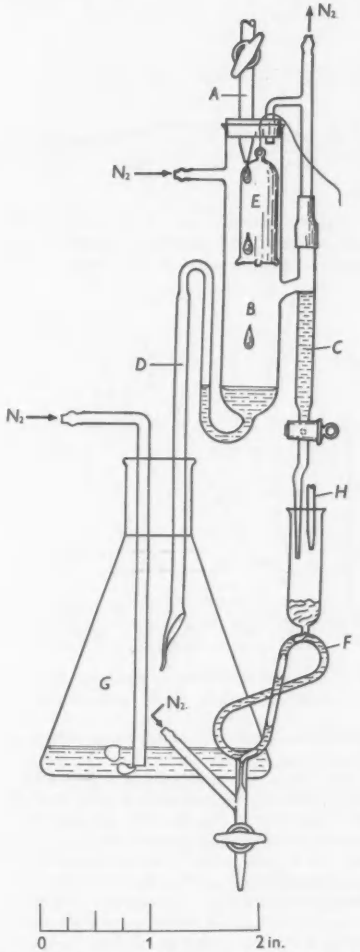

Fig. 1. Siphon arrangement for collection and titration of chromatographic column eluate. (Showing siphon having drained, leaving titration sample in side arm.) *A*, tap of chromatographic column; *B*, main receiver (21 ml.). *C*, side arm for collection of sample for titration, 2·75 ml.; *D*, siphon; *E*, plunger for altering sample volume by raising or lowering; *F*, titration vessel with N_2 circulation (cf. Howard & Martin, 1950); *G*, receiver for collection of main bulk of eluate under N_2; *H*. burette tip.

Preliminary stages of isolation

Lithium salt–acetone separation. Total pilchard acids (300 g., average equiv. wt., 282; average number of double bonds per molecule, 2·40) were subjected to lithium soap–acetone segregation twice in 95% aqueous acetone and finally in 97% aqueous acetone (cf. Silk *et al.* 1954). A concentrate (93 g.) of unsaturated acids was obtained which had an average equiv. wt., 307 and an average number of double bonds per molecule 4·55 (H_2 uptake Pd–$BaSO_4$).

Urea complex fractionation. The above material was fractionated further, using the urea complex method (cf. Silk *et al.* 1954). A non-complex forming concentrate of very highly unsaturated acids was obtained having an average equiv. wt., 308 and an average number of double bonds per molecule, 4·85 (H_2 uptake Pd–$BaSO_4$).

Molecular distillation of the unsaturated acid concentrate. The molecular still described by Sutton (1953) was used to distil the concentrate of unsaturated acids obtained by urea complex fractionation. All distilling stages were maintained at the same temperature and the distilland was recycled twice before removal of each fraction. Successive fractions were obtained using solvents of increasing boiling point to heat the distilling stages. The lowest boiling fraction (*A*) which distilled at 85° contained the required C_{16} tetraene acid in the highest concentration and was reserved for subsequent chromatography. Results are shown in Table 1.

Reversed-phase partition chromatography of unsaturated acid concentrate on a preparative scale

A portion of the above fraction *A* was chromatographed on a column 80 cm. high × 3 cm. diameter which was maintained at 10° and packed with non-wetting kieselguhr supporting medicinal paraffin as stationary phase (cf. Silk & Hahn, 1954). The mixed acids (413 mg.) were dissolved in aqueous acetone (55%, v/v previously equilibrated with paraffin), and the solution was allowed to run into the top of the column. Last traces of acid were washed in with 20 ml. 55% aqueous acetone. The chromatogram was then developed at a flow rate of 150 ml./hr. with 55% aqueous acetone (pre-cooled to 10°).

The column eluate was collected under N_2 in a siphon constructed such that each sample was divided into two portions; the main bulk and a smaller sample which was titrated for the purpose of following the course of chromatographic resolution (Fig. 1). Methanolic 0·01 N-KOH solution with bromothymol blue (0·2% in 70% aqueous acetone) as indicator, was used for titration of samples in a circulating vessel similar to that described by Howard & Martin (1950). The results for fraction *A* are shown in Fig. 2.

Recovery of hexadecatetraenoic acid from chromatographic column eluate

The column eluate representative of the first peak shown on chromatographic resolution of fraction *A* (that between the two arrows on Fig. 2) was collected separately. The

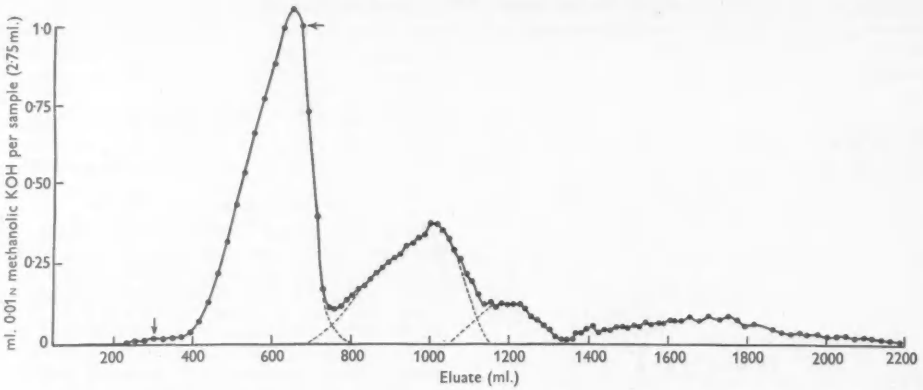

Fig. 2. Chromatographic resolution of fraction *A* (preparative scale). Weight of fraction *A*, 413 mg.; column: 80 × 3 cm. Temp. 10°; Flow rate, 150 ml./hr. Solvent system: aqueous acetone (55%, v/v)–medicinal paraffin supported on non-wetting kieselguhr. Eluate between arrows collected separately as representative of first peak.

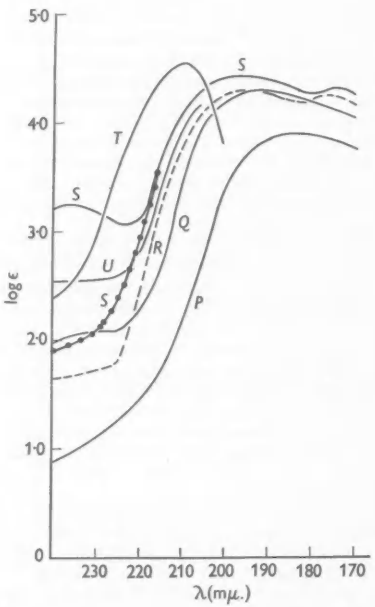

Fig. 3. Ultraviolet spectra of fatty acids in the region 170–240 mμ. *P*, oleic acid; *Q*, linoleic acid; *R*, linolenic acid; *S*, methyl arachidonate. (These curves are from Rusoff, Platt, Klevens & Burr (1945).) *S* (●–●) methyl arachidonate in region 220–240 mμ. from Barnes, Rusoff, Miller & Burr (1944). *T*, heptadec-2-enoic acid from Lauer, Gensler & Miller (1941). *U*, hexadecatetraenoic acid isolated from pilchard oil.

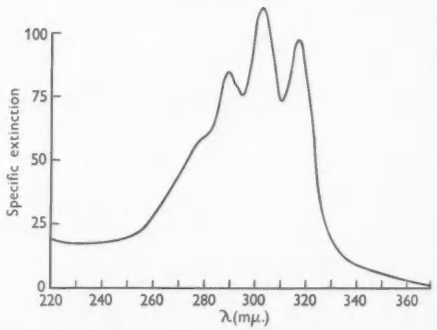

Fig. 4. Ultraviolet absorption of alkali-isomerized hexadecatetraenoic acid. (For details see text.)

solution (350 ml.) was evaporated to approximately half its volume at 35° and 15 mm. under N_2.

The resulting aqueous suspension of fatty acid was then saturated with Na_2SO_4 and exhaustively extracted with distilled *n*-pentane under N_2. The pentane extract was washed with water and evaporated to yield the fatty acid (144 mg.) as a pale yellow (almost colourless) oil of refractive index n_D^{29} 1·4870. (Found: C, 77·1, H, 9·9%. No. of double bonds/mol., 3·96 (H_2 uptake over Adams's catalyst); equiv. wt., 250. $C_{16}H_{24}O_2$ (*hexadecatetraenoic acid*) requires C, 77·3; H, 9·7%. No. of double bonds/mol., 4·0, equiv. wt., 248·5.)

Hydrogenation product. Recovery of the hydrogenation product, followed by crystallization from 96% ethanol gave a fatty acid of equiv. wt., 258 and m.p., 62·0–62·5°. (Palmitic acid has equiv. wt. = 256·4 and m.p. 62·85°.) Mixed m.p. with an authentic sample of palmitic acid (m.p. 62·3–62·6°) showed a m.p. of 62·1–62·6°, thus confirming that the hydrogenation product was palmitic acid.

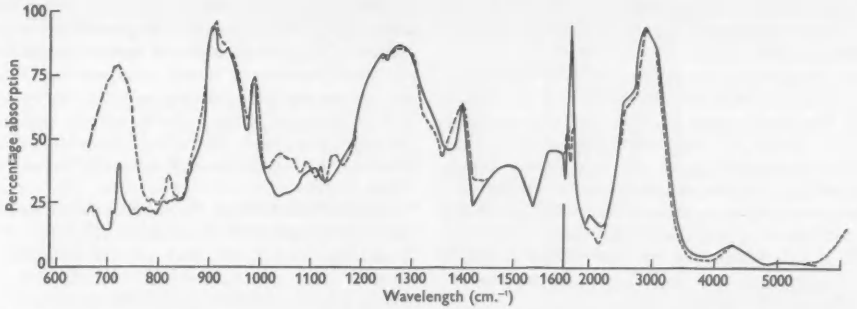

Fig. 5. Infrared absorption of hexadecatetraenoic acid (smooth curve 3·04%, w/w, CS_2) and undec-10-enoic acids (broken curve), 2·47%, w/w, CS_2.

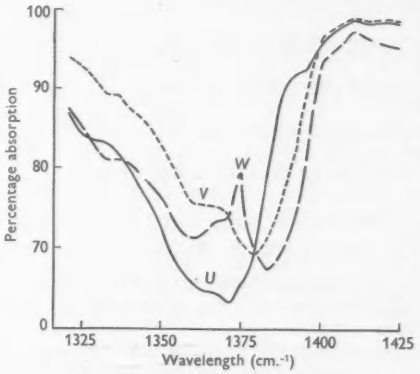

Fig. 6. Infrared absorption 1325–1425 cm.$^{-1}$ in CCl_4 of hexadecatetraenoic acid (4·97%, w/w, *U*) undec-10-enoic acid (5·00%, w/w, *V*) and palmitic acid (4·84%, w/w, *W*).

Ultraviolet absorption. The ultraviolet absorption curve of the hexadecatetraenoic acid showed no peaks in the region 220–320 mμ. A faint inflexion at 231 mμ. indicated that a negligible amount of oxidation had taken place. Comparison of the $E_{1\%}^{1\,cm.}$ observed at 231 mμ. (1·95) with that of pure methyl linoleate hydroperoxide (780) at the same wavelength (Sephton & Sutton, 1953) showed that the maximum amount of oxidation that could have taken place was 0·33%.

The ultraviolet absorption of the hexadecatetraenoic acid in the region 206–240 mμ. is shown in Fig. 3, together with that of several other acids in the far ultraviolet region (170–240 mμ.)

Alkali isomerization. This was carried out according to the method of Herb & Riemenschneider (1952) by heating a 13 mg. sample for 15 min. at 180° in 21% KOH–glycol reagent. The ultraviolet absorption of the product is shown in Fig. 4.

Infrared absorption. The infrared absorption curve of the hexadecatetraenoic acid in CS_2 is shown together with that of undec-10-enoic acid in Fig. 5. The infrared absorption of hexadecatetraenoic, undec-10-enoic and palmitic acids in CCl_4 in the region 1325–1425 cm.$^{-1}$ is illustrated on a larger scale in Fig. 6.

Permanganate oxidation of hexadecatetraenoic acid and paper chromatography of the oxidation products

Oxidation of the hexadecatetraenoic acid with cold alkaline $KMnO_4$ yielded adipic acid and a little glutaric acid.

A sample of the hexadecatetraenoic acid was oxidized at 0° with cold alkaline $KMnO_4$, according to the method of Dolby, Nunn & Smedley-Maclean (1940). Removal of the monobasic acid products by steam distillation followed by continuous ether extraction of the (HCl) acidified residue, furnished the dibasic oxidation products as a crystalline mass after evaporation of the ether. The dibasic products were spotted on to sheets of Whatman no. 1 paper, together with reference C_2–C_6 dicarboxylic acids, and the chromatograms developed with NH_3 (sp.gr. 0·88)–ethanol (20:80, v/v), according to Long, Quayle & Stedman (1951). Development was carried out in a descending direction for 14 hr. at 30° and the solvent allowed to run off the bottom of the paper. The papers were dried for 45 min. at 80° and sprayed with British Drug Houses Universal Indicator to contrast the acid spots. All spots were separated by at least 1·5 cm., and the major product of oxidation was shown to be adipic acid by its exact correspondence with a spot of authentic adipic acid. Some glutaric acid was also evidenced by a spot clearly resolved from the adipic acid. Several chromatograms were carried out with the same result.

DISCUSSION

Ultraviolet evidence shows that the hexadecatetraenoic acid isolated from pilchard oil does not contain conjugated double bonds, since no diene, triene or tetraene peaks were apparent in the region 220–320 mμ. Comparison of the ultraviolet absorption with that of known acids in the region 170–240 mμ. (Fig. 3) shows that the curve lies in the expected position close to that of methyl arachidonate (C_{20} tetraene ester). The molar absorption observed at 210 mμ. is about one-twentieth of that shown by the peak of heptadec-2-enoic acid which

has unsaturation conjugated with the carboxyl group. The hexadecatetraenoic acid cannot therefore be $\alpha\beta$-unsaturated.

The ultraviolet absorption of the alkali-isomerized hexadecatetraenoic acid (Fig. 4), shows clearly the three peaks of a conjugated tetraene. No other peaks are apparent. The acid is thus probably a tetraene with all the double bonds separated by a single methylene group. If this were not so, then tetraene absorption would not have been evidenced after alkali isomerization because double bonds separated by more than a single methylene group are unlikely to isomerize into conjugation (cf. Farmer, 1942).

Only five structures are possible for a C_{16} tetraene acid in which the double bonds are separated by a single methylene group. These are as follows:

$HO_2C.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_3$ I
2:5:8:11-Tetraene

$HO_2C.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_3$ II
3:6:9:12-Tetraene

$HO_2C.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_3$ III
4:7:10:13-Tetraene

$HO_2C.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_3$ IV
5:8:11:14-Tetraene

$HO_2C.CH_2.CH_2.CH_2.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH_2$ V
6:9:12:15-Tetraene

$HO_2C.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH{:}CH_2$ VI

$HO_2C.CH{:}CH.CH{:}CH.CH{:}CH.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.CH{:}CH_2$ VII

Of these structures (I) is $\alpha\beta$-unsaturated and is excluded by the ultraviolet evidence. Structures I–IV all contain a C-CH_3 group which exhibits a well defined peak at approximately 1375 cm.$^{-1}$ in the infrared. No such peak was apparent in the infrared curve of the hexadecatetraenoic acid (cf. Fig. 6, which shows that the C-CH_3 absorption of palmitic acid is completely absent from the hexadecatetraenoic acid).

Structures I–IV are thus clearly impossible for the isolated acid, which can therefore most probably be represented by structure V containing a terminal vinyl group. This grouping exhibits intense maxima at 910 and 990 cm.$^{-1}$ in the infrared, and both these peaks were strongly evidenced in the absorption curve of the hexadecatetraenoic acid (Fig. 5). The spectrum of undec-10-enoic acid, which contains the —$CH{:}CH_2$ grouping, was used as a reference curve.

trans-trans Conjugated double bonds such as might arise from autoxidation of an unsaturated acid, also exhibit an infrared maximum at 990 cm.$^{-1}$, but since no peak due to oxidation was shown by the ultraviolet absorption curve of the isolated acid, there is no likelihood that the observed peak at 990 cm.$^{-1}$ can have been due to oxidation. The measured intensity of this peak, if due to *trans-trans* conjugated bonds and not to a terminal vinyl group, would correspond to some 50 % oxidation having taken place, which is clearly impossible.

The validity of structure V is also confirmed by the identification of adipic acid among the permanganate oxidation products of the isolated acid. The presence of some glutaric acid in addition is probably the result of further degradation of the adipic acid (cf. Begemann, Keppler & Boekenoogen, 1950).

The related structure VI which includes a terminal vinyl group and which would also give adipic acid on oxidation, cannot be that of the isolated acid, because the conjugated triene-carboxylic acid which might arise on alkali isomerization (VII) would only show one peak at approx. 300 mμ. in the ultraviolet (cf. Hauser, Kuhn, Smakula & Hoffer, 1935, and Weedon & Woods, 1951). Since the three peaks of a conjugated tetraene were observed for the alkali-isomerized acid (Fig. 4), structure VI cannot obtain.

Thus structure (V), hexadeca-6:9:12:15-tetraenoic acid, is clearly established as that of the acid isolated from pilchard oil. This compound, which has not previously been reported, occurs to an extent of approximately 1·3 % of the total pilchard acids.

During the course of preparation of this paper it has come to our notice that a C_{16} tetraene acid has recently been isolated by Toyama & Yamamoto (1953) from Japanese sardine oil. The acid was insufficiently characterized, but one of the two possible structures suggested contained a terminal vinyl group. A second structure in which the double bonds occupy the positions 4, 8, 11, 14 was preferred by these authors, on the grounds that succinic acid was isolated after oxidative fission.

SUMMARY

1. The techniques of lithium soap–acetone segregation, molecular distillation, urea complex fractionation and reversed-phase partition chromatography have been applied in successive subdivision of the total fatty acids of South African pilchard oil.

2. Application of these techniques has resulted in the isolation of a new acid characterized by physical and chemical means as hexadeca-6:9:12:15-tetraenoic acid.

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Studies in the Biochemistry of Micro-organisms

93. CYCLOPENIN, A NITROGEN-CONTAINING METABOLIC PRODUCT OF *PENICILLIUM CYCLOPIUM* WESTLING

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The *Penicillium cyclopium* series of species of *Penicillium* contains four accepted species and one variety, i.e. *P. cyclopium* Westling, *P. cyclopium* Westling var. *echinulatum* Raper & Thom, *P. puberulum* Bainier, *P. martensii* Biourge and *P. aurantio-virens* Biourge. The series has proved to be a fruitful source of mould metabolic products, the following having been described: penicillic acid from *P. puberulum* (Alsberg & Black, 1913) and *P. cyclopium* (Birkinshaw, Oxford & Raistrick, 1936); ergosterol in the mycelium of *P. puberulum* (Birkinshaw, Callow & Fischmann, 1931); the tropolones, puberulic and puberulonic acids, from *P. puberulum* and *P. aurantio-virens* (Birkinshaw & Raistrick, 1932) and *P. johannioli* Zaleski = *P. martensii* Biourge (Oxford, Raistrick & Smith, 1942); *i*-erythritol from *P. cyclopium* (Oxford & Raistrick, 1935); the anthraquinones, ω-hydroxyemodin and emodic acid, from *P. cyclopium* (Anslow, Breen & Raistrick, 1940); cyclopolic and cyclopaldic acids, two fully substituted benzoic acids, from *P. cyclopium* (Birkinshaw, Raistrick, Ross & Stickings, 1952) and an unnamed photosensitive nitrogenous compound, $C_{17}H_{12}O_2N_2$, m.p. 220° (decomp.), from *P. puberulum*, which gave phenol on heating and *p*-hydroxybenzoic acid on oxidation (Campbell, Foss, Hirst & Jones, 1945).

We now wish to report the isolation from culture filtrates of an authentic strain of *P. cyclopium* of a hitherto undescribed metabolic product for which we propose the name cyclopenin.

CYCLOPENIN

Cyclopenin was isolated, in roughly equal amounts, from culture filtrates of *P. cyclopium*, L.S.H.T.M. no. 72, grown on either Czapek-Dox or Raulin-Thom solutions, by adsorption on charcoal and elution with methanol. Cyclopenin, $C_{17}H_{14}O_3N_2$, crystallizes from methanol in colourless needles containing two molecules of water of crystallization. On heating, these crystals melt at 110–120° with frothing and loss of water, reset on cooling and remelt at 207°. Cyclopenin separates from a variety of other solvents, especially ethyl acetate, in crystals containing half a molecule of water of crystallization which melt sharply at 207°. It

contains one nitrogen-methyl group and has 1·5 atoms of active hydrogen, but has no methoxyl or carbon-methyl groups. It is strongly laevorotatory in ethanol solution. It dissolves only sparingly in cold water, does not dissolve readily in aqueous sodium bicarbonate but is easily soluble in dilute aqueous sodium hydroxide. Addition of bromine water to its saturated aqueous solution gives an immediate thick turbidity which readily flocks to a heavy precipitate under acid conditions. It shows slight antibacterial activity towards *Micrococcus pyogenes* var. *aureus* and *Escherichia coli*. It does not give a ferric colour either in aqueous or ethanolic solution, except on long standing.

It is readily decomposed, even in the cold, by dilute mineral acids with the formation of one molecule each of carbon dioxide, methylamine and viridicatin according to the equation

$$\underset{\text{cyclopenin}}{C_{17}H_{14}O_3N_2} + H_2O \rightarrow CO_2 + CH_3NH_2 + \underset{\text{viridicatin}}{C_{15}H_{11}O_2N}$$

Viridicatin

Viridicatin was first described by Cunningham & Freeman (1953), who isolated it from the dried mycelium of a strain of *Penicillium viridicatum* Westling by chloroform extraction. *P. viridicatum*, although not included in the *P. cyclopium* series is closely related to this species, so that strains of the two species are often difficult to distinguish. Viridicatin, $C_{15}H_{11}O_2N$, forms colourless needles, m.p. 268°, which are optically inactive in ethanol and give an intense green ferric colour in ethanol solution. Cunningham & Freeman described seven crystalline derivatives of viridicatin but lacked sufficient material for degradative work, since the mould ceased to produce viridicatin after being in cultivation for some time.

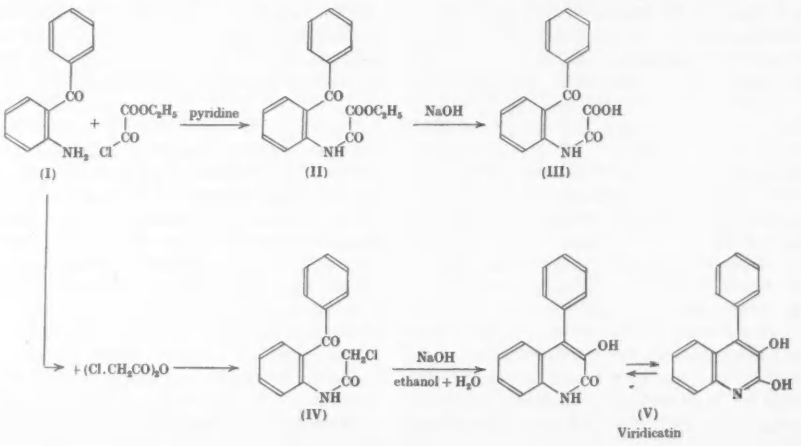
We have now found that, although we were unable to detect viridicatin in the mycelium of strain no. 72 of *P. cyclopium*, which produces cyclopenin, five other strains of this species give considerable amounts of viridicatin. Such strains can be readily detected by growing them on Czapek-Dox 5% glucose solution for 21–24 days. Portions of the fresh mycelium are pressed as dry as possible between sheets of filter paper and are then extracted for a short time with boiling ethanol. Production of a strong green colour on addition of ferric chloride to the filtered ethanolic extract is strong presumptive evidence of the presence of viridicatin.

We have confirmed Cunningham & Freeman's proposed formula, $C_{15}H_{11}O_2N$, for viridicatin and have prepared and described the following new derivatives: 3:5-dinitrobenzoic acid salt, $C_{15}H_{11}O_2N.C_7H_4O_6N_2$, yellow prisms, m.p. 238°; monoacetylviridicatin, $C_{17}H_{13}O_3N$, short colourless prisms, m.p. 200–201°; *OO*-dimethylviridicatin, $C_{15}H_9N.(OCH_3)_2$, colourless plates, m.p. 86–87°; *ON*-dimethylviridicatin, $C_{15}H_9O.(OCH_3)(NCH_3)$, colourless plates, m.p. 197–198°.

Work carried out in this laboratory on the degradation of viridicatin has led to the establishment of structure (V) for it, and this structure has been confirmed by synthesis. It has been shown that when oxygen is bubbled at room temperature through a solution of viridicatin in ethanolic potassium hydroxide, oxidative decomposition of viridicatin takes place with the formation in high yield of *o*-aminobenzophenone (I) and oxalic acid according to the equation

$$C_{15}H_{11}O_2N + H_2O + O_2 \rightarrow C_{13}H_{11}ON + (COOH)_2.$$

Further, when viridicatin was oxidized with potassium permanganate in boiling acetone solution, or with hydrogen peroxide in glacial acetic acid solution



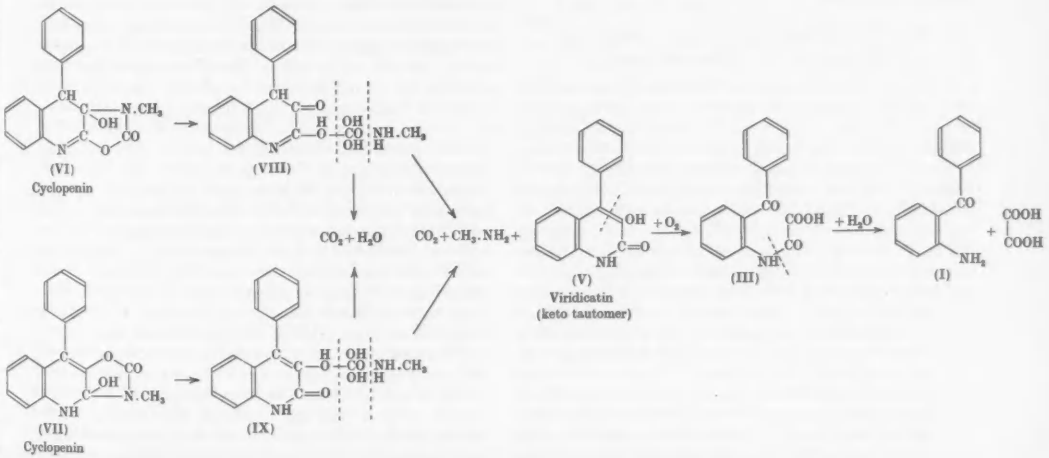
at 100°, a good yield of the hitherto unknown oxalyl-*o*-aminobenzophenone (III) was obtained. This compound, which crystallizes in colourless needles, m.p. 184° (decomp.), was synthesized as follows: *o*-aminobenzophenone (I), in pyridine solution, was condensed with ethoxalyl chloride. The resulting ester, ethoxalyl-*o*-aminobenzophenone (II), colourless irregular plates, m.p. 135–136°, was hydrolysed at room temperature with aqueous N sodium hydroxide giving an 81 % overall yield of oxalyl-*o*-aminobenzophenone (III) identical with that obtained by permanganate oxidation of viridicatin.

It is therefore very probable that the oxidation of viridicatin in alkaline solution with gaseous oxygen proceeds, through the formation and action of peroxides, to give oxalyl-*o*-aminobenzophenone (III) which is then hydrolysed by the alkali to give oxalic acid and *o*-aminobenzophenone (I).

Viridicatin was synthesized by a modification of the method of Camps (1899, 1902) for the synthesis of carbostyrils (2-hydroxyquinolines). *o*-Aminobenzophenone was chloroacetylated by treatment with chloroacetic anhydride. The resulting chloroacetyl *o*-aminobenzophenone (IV), colourless needles, m.p. 107–108°, was heated in aqueous ethanol with sodium hydroxide. The hot solution was then acidified with hydrochloric acid. The cyclization product (V) separated in 72 % overall yield as colourless needles, which, after crystallization from ethanol, melted at 268° not depressed on admixture with natural viridicatin of the same melting point. The *ON*-dimethyl derivative of synthetic viridicatin, that of natural viridicatin and a mixture of the two all melted at 197°.

Viridicatin is thus 2:3-dihydroxy-4-phenylquinoline or its keto tautomer.

The solution of the problem of the structural formula for cyclopenin therefore resolves itself into that of piecing together one molecule each of carbon dioxide, methylamine and 2:3-dihydroxy-4-phenylquinoline (V), with the loss of one molecule of water, in such a way as best to illustrate the known properties of cyclopenin. In particular, it must account for the optical activity of cyclopenin, the ready formation of the three hydrolytic products of cyclopenin mentioned above and the formation of the oxidative hydrolytic products of viridicatin, i.e. oxalic acid, oxalyl-*o*-aminobenzophenone (III) and *o*-aminobenzophenone (I). To this end we suggest for cyclopenin either structure (VI) or (VII) with a slight preference for (VI), although we have no evidence which conclusively supports one structure against the other. Both structures satisfactorily explain the optical activity of cyclopenin and the ready formation of its hydrolytic products through the intermediate formation either of the 2-methylurethane (VIII from VI) or the 3-methylurethane (IX from VII) of 2:3-dihydroxy-4-phenylquinoline (V), either of which, on acid or alkaline hydrolysis, would be expected to yield carbon dioxide, methylamine and viridicatin (V). When viridicatin (and therefore almost certainly cyclopenin) is submitted to oxidative hydrolysis, e.g. by gaseous oxygen, in alkaline solution, potassium permanganate in acetone or hydrogen peroxide in glacial acetic acid, the initial oxidation product is oxalyl-*o*-aminobenzophenone (III) which is then readily hydrolysed to give oxalic acid and *o*-aminobenzophenone (I). All these changes are illustrated in the following scheme:



EXPERIMENTAL

CYCLOPENIN

History of culture

The culture used for the preparation of cyclopenin was isolated by Mr R. L. Hughes from a catch plate exposed by him in a laboratory in Croydon, Surrey. The culture, which bears Mr Hughes's catalogue no. 26, was received by one of us (A. B.) in March 1947 and was given the London School of Hygiene catalogue no. 72. The following note on the culture was kindly supplied by Mr George Smith of this Department. 'I have examined the *Penicillium* no. 72 and find that it is a typical strain of *P. cyclopium* Westling. Colonies on Czapek agar are bright blue-green, with little colour in reverse, spreading, granular and showing well-marked fasciculation, particularly in the younger areas. Conidiophores are slightly rough, and the conidia round, smooth, about 3 μ. in diameter. These characteristics alone are sufficient to place the mould with certainty as *P. cyclopium*. This species is probably the commonest member of the genus in this country, growing on a very wide range of substrata, so that it is hardly surprising that different isolates vary in their biochemical characteristics.'

Sand cultures of *P. cyclopium* strain no. 72 were prepared, for preservation, as follows. Acid-treated, washed and dried sand was sterilized by heating at 140–150° for 4–6 hr. Portions of this sterile sand were added to each of a number of 11-day-old cultures of *P. cyclopium* no. 72 grown at 24° on malt-agar slopes. The cultures were thoroughly shaken and the sand acquired a greenish tint from adhering spores. The sand and spores were transferred aseptically to sterile test tubes and dried for some days *in vacuo* over P_2O_5. Each tube, still plugged with sterile cotton wool, was drawn out in a flame to a capillary some distance below the cotton-wool plug. After cooling, each tube was evacuated for 5–10 min. on an oil pump, hermetically sealed at the capillary while still under vacuum, and stored in the dark at room temperature. Sand cultures so prepared in February 1948 were still highly viable in October 1953 and, after cultivation on malt-agar slopes at 24°, furnished cultures which gave good yields of cyclopenin.

Cultural conditions and cultural characteristics of Penicillium cyclopium *strain no.* 72

(*a*) *On Raulin-Thom solution.* Glucose, 75·0 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4.7H_2O$, 0·07 g.; $ZnSO_4.7H_2O$, 0·07 g.; distilled water, 1500 ml. This solution was distributed into fifty 1 l. conical flasks (350 ml./flask), which were plugged with cotton wool and sterilized. Each flask was inoculated with a portion of a spore suspension, in sterile distilled water, of *P. cyclopium* strain no. 72 which had been cultivated on 12 malt-agar slopes at 24° for 10–12 days. The inoculated flasks were incubated at 24° in the dark. The mould was somewhat slow to germinate, but after 4 days the surface of the medium in most of the flasks was practically covered with a thin, white growth showing occasional green patches of sporing growth. After 6 days the medium in almost all flasks was covered with a somewhat fragile bright parrot-green felt which had an orange or pinkish orange reverse. The culture solution was now bright orange in colour. With continued cultivation the felts became somewhat bluer in tone and from 14 days onwards became much duller in appearance and wrinkled and buckled in texture.

(*b*) *On Czapek-Dox solution.* Glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1000 ml. The cultural conditions were the same as for the cultures on Raulin-Thom solution in (*a*) above. Growth in the early stages was similar to that on Raulin-Thom solution except that after 4 days, when the mould was beginning to spore in patches, there was much yellow growth, and after 8 and 15 days the mycelium was a much duller green in shade than the bright parrot-green of the Raulin-Thom cultures. There was also no marked orange colour in the mycelial reverse and the culture solution was only pale yellow.

At the end of the incubation period the culture filtrates obtained from culture solutions (*a*) and (*b*) gave very similar reactions which differed chiefly in intensity. Addition of aqueous $FeCl_3$ solution did not give an immediate colour reaction, although an emerald-green colour slowly developed in the course of 3–4 days. An intense emerald-green colour was however immediately produced on the addition of aqueous $FeCl_3$ to a portion of the culture filtrate which had been acidified with HCl, heated at 100° for 20 min., cooled and neutralized with aqueous NaOH. As will be seen later this is due to the hydrolysis of the metabolic product (cyclopenin), which itself gives no ferric colour, with the formation of a product (viridicatin) which immediately gives the characteristic ferric colour. Addition of saturated Br_2 water to either culture filtrate leads to absorption of Br_2 and the formation of a thick turbidity which slowly coagulates to a heavy, pale yellow, amorphous precipitate. If the culture filtrate is acidified with dilute HCl before the addition of Br_2 water a heavy precipitate is formed almost immediately.

Isolation of cyclopenin from culture filtrates of Penicillium cyclopium *strain no.* 72

Cultures of *P. cyclopium* strain no. 72, grown on either Raulin-Thom solution or on Czapek-Dox solution, were harvested after 14–17 days and 21–22 days respectively. The culture fluid from fifty flasks was decanted from the mycelium and filtered through a thin layer of kieselguhr on a large Büchner funnel. The mycelium was pressed as thoroughly as possible in a tincture press, dried in a vacuum oven at 40–45° and weighed. The filtered pressings were added to the main filtrate and the solution was adjusted to about pH 2·0 with conc. HCl. Activated charcoal (50–60 g.) (British Drug Houses Ltd., London) was then added. The mixture was stirred vigorously for 1–2 hr. when a sample, freed from charcoal by filtration, should be quite colourless, give no turbidity with Br_2 water and no colour with aqueous $FeCl_3$ after hydrolysis with HCl. The charcoal was separated by filtration through a thin layer of kieselguhr (10 g.) on a 24 cm. Büchner filter and washed with distilled water (1–1·5 l.) till the washings were free from chlorides. It was drained as thoroughly as possible, dried in the open laboratory overnight and then thoroughly dried in a vacuum desiccator over conc. H_2SO_4. The charcoal was suspended in methanol (500 ml.) and poured on to a preformed kieselguhr pad on a filter disk at the foot of a long, wide, glass column. A total of 3–3·5 l. of methanol was then passed through the column under a very slight vacuum, thus eluting all the desired product. The methanol solution was concentrated *in vacuo* to about 40 ml., when cyclopenin began to separate

Table 1. *Cultural details and yields of metabolic products from* Penicillium cyclopium *strain no.* 72

Each batch contains fifty flasks. For other details see text.

	Raulin-Thom solution				Czapek-Dox solution			
Batch no.	*R*1	*R*2	*R*3	*R*4	*C*1	*C*2	*C*3	*C*4
Incubation period (days)	17	14	14	14	22	21	22	22
Mycelial dry weight (g.)	154	195	193	192	134	138	—	154
Glucose (% by polarimeter)	0·17	0·45	0·60	0·44	0·82	0·76	0·63	0·52
pH	4·4	3·9	3·6	3·9	4·9	4·6	4·6	4·6
Crystalline cyclopenin (g.)	3·50	4·42	4·50	2·15	3·39	3·36	2·71	2·80
Residual gum (g.)	6·45	6·64	6·96	7·00	6·64	7·00	10·10	6·69
Total extract (g.)	9·95	11·06	11·46	9·15	10·03	10·36	12·81	9·49

and was collected by filtration. The cyclopenin so obtained melted at 106–110° with evolution of water of crystallization, then reset and remelted at 207°. By evaporation of the filtrate other crops of crystals were obtained. The final mother liquors, on evaporation to dryness, gave a light orange residual gum.

Cyclopenin is produced on both Raulin-Thom and Czapek-Dox media in comparable amounts, but we prefer to use Raulin-Thom medium, since the incubation period is about 7 days shorter, the yield appears to be more constant and cyclopenin is obtained as beautiful snow-white needles from Raulin-Thom medium, whereas they are just off colourless with Czapek-Dox medium. The yield of cyclopenin and a number of cultural details are given in Table 1.

Purification of cyclopenin

Most of the specimens of cyclopenin listed in Table 1 as crystalline are already sufficiently pure for preparative work. Cyclopenin may, however, be further purified by recrystallization from a variety of solvents. Thus 3·2 g. cyclopenin, dissolved in 25 ml. of boiling methanol, yielded 1·90 g. of colourless needles containing 2 molecules of water of crystallization (see below), which melt with frothing and loss of water at 110–120°. The melt resets on cooling in well formed crystals which, on reheating, melt at 207°. Cyclopenin also crystallizes well from ethyl acetate (0·55 g. in 15 ml.) and ethanol (1·2 g. in 10 ml.). It separates from both these solvents in colourless tablets, m.p. 207° with little decomposition, which are less soluble in most solvents than the crystals from methanol.

General properties of cyclopenin

Cyclopenin contains C, H, O and N, but not S, Cl or P. (Found (*a*) on sample crystallized from methanol and air dried: C, 62·2, 62·4; H, 5·5, 5·4; N, 8·4, 8·5. $C_{17}H_{14}O_3N_2$, $2H_2O$ requires C, 61·8; H, 5·5; N, 8·5%. (*b*) On sample crystallized from methanol and dried to constant weight *in vacuo* at room temp.: C, 66·9; H, 4·9; N, 9·2; OMe, nil. $C_{17}H_{14}O_3N_2$, $\frac{1}{2}H_2O$ requires C, 67·3; H, 5·0; N, 9·2%. (*c*) On different samples crystallized from ethyl acetate and dried in high vacuum, without loss in weight, either at room temp. or at 60–65°: C, 66·9, 67·1; H, 4·9, 4·65; N, 9·1 (Dumas), 9·2, 9·3 (Kjeldahl); OMe, nil; *C*-Me, nil; *N*-Me, 4·0; active H, 0·54%; mol.wt., cryoscopic in camphor, 300. $C_{17}H_{14}O_3N_2$, $\frac{1}{2}H_2O$ requires C, 67·3; H, 5·0; N, 9·2; *N*-Me, 5·0; 1·5 active H, 0·50%; mol.wt. 303.)

It is laevo-rotatory in ethanol solution and shows no sign of mutarotation, the rotation remaining unchanged overnight. (Found (*a*) on sample crystallized from methanol and containing $2H_2O$ of crystallization: $[\alpha]^{18}_{5461}$ −280°, $[\alpha]^{18}_{5790}$ −240° in ethanol (*c*, 2·000). (*b*) On sample crystallized from ethyl acetate and containing $\frac{1}{2}H_2O$ of crystallization: $[\alpha]^{20}_{5461}$ −306°, $[\alpha]^{20}_{5790}$ −263° in ethanol (*c*, 1·000). A solution of cyclopenin (0·50 g. of the dihydrate) in ethanol (15 ml.) and aqueous N-HCl (75 ml.), which showed an initial rotation in a 1 dm. tube of −1·52° in the Hg green (5461 Å) light, slowly became optically inactive at room temp., the following rotations being observed at the stated times: 1·75 hr., −1·40°; 3·25 hr., −1·20°; 5·5 hr., −1·05°; 23 hr., −0·44°; 96 hr., −0·10°; 102 hr., 0·00°. Viridicatin separated as colourless crystals (0·27 g., m.p. 248–252°, raised to 268° on recrystallization from ethanol).

Cyclopenin does not dissolve in aqueous $NaHCO_3$. It is readily soluble in N-NaOH, but is precipitated on neutralizing with HCl and redissolves in excess of HCl. Cyclopenin does not reduce Fehling's solution, even on boiling. Addition of Br_2 water to a saturated aqueous solution of cyclopenin gives an immediate thick turbidity which does not flocculate rapidly. If, however, Br_2 water is added to a cyclopenin solution acidified with HCl an immediate heavy precipitate is formed. Aqueous $FeCl_3$ gives no immediate colour with an ethanolic cyclopenin solution. After standing for 24 hr. a pale emerald-green colour develops, becoming intense emerald green after some days and red on dilution with much tap water. The development of an intense green ferric colour is almost certainly due to hydrolysis, since viridicatin, which is one of the hydrolytic products of cyclopenin, gives an immediate intense emerald-green ferric colour. Cyclopenin is readily hydrolysed, with the formation of viridicatin, by treatment at room temperature with conc. H_2SO_4 or conc. HCl, or by boiling with aqueous N-NaOH or 2N-H_2SO_4.

A sterile solution of cyclopenin (40·0 mg.) in ethanol (0·5 ml.) was added to sterile nutrient broth (9·5 ml.) thus giving a concentration of cyclopenin in the broth of 1:250. Serial dilutions with sterile nutrient broth were then made and inoculated with either *Micrococcus pyogenes* var. *aureus* (Oxford H strain) or *Escherichia coli* (National Collection of Type Cultures R. 6). The tubes were incubated at 37° for 24 hr. when there was complete inhibition of the growth of *E. coli* at a concentration of cyclopenin of 1:500, partial inhibition at 1:1000 and no inhibition at 1:2000; partial inhibition of *M. pyogenes* at 1:500 and no inhibition at 1:1000.

Attempts to prepare a crystalline acetyl derivative of cyclopenin failed, either with acetic anhydride and pyridine at room temp. or with acetic anhydride and anhydrous sodium acetate at 100°.

Quantitative hydrolysis of cyclopenin with 2N-HCl, *giving* CO_2, *methylamine and viridicatin* (V)

A suspension in 2N-HCl (100 ml.) of cyclopenin hydrate (1·981 g.; m.p. 207°; recryst. from ethyl acetate), contained in a two-necked flask fitted with an air condenser, was gently boiled in an oil bath in a stream of CO_2- and O_2-free N_2. The effluent N_2 was scrubbed first through a bubbler containing Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in aqueous 2N-HCl) and then through two bubblers, each containing 50·00 ml. of 0·2N-$Ba(OH)_2$. Evolution of CO_2 began quickly and was completed in 2·5 hr. when the hydrolysis was stopped. The contents of the baryta bubblers were then titrated against 0·1N-HCl. The observed titre was 120·2 ml. 0·1N-HCl, equivalent to 92·0% of 1 mole CO_2/mole cyclopenin.

No trace of precipitate was obtained in the Brady bubbler indicating the absence, as hydrolytic products, of any volatile aldehyde or ketone.

The hydrolysis solution was held at 0° overnight and the colourless solid (crude viridicatin) which separated was collected quantitatively, washed with water and dried (1·431 g., m.p. 246–252°, corresponding to 92·3% of 1 mole of viridicatin/mole of cyclopenin). A portion of the crude viridicatin was carefully purified by sublimation in high vacuum at 160–170° followed by repeated crystallization from ethanol. It was finally obtained as colourless, fine, shining needles, m.p. 268° not depressed on admixture with a specimen of viridicatin isolated from *P. cyclopium* strain no. 578. (Found: C, 76·0; H, 4·7; N, 6·0. Calc. for $C_{15}H_{11}O_2N$: C, 75·9; H, 4·7; N, 5·9%.)

The combined acid filtrate and washings from the crude viridicatin were now distilled *in vacuo*, distilled water being run in at about the same rate as the distillate came over. Titration of the distillate showed that no volatile organic acids had been formed during the hydrolysis.

The combined acid filtrate and washings remaining after the vacuum distillation described in the previous paragraph were now evaporated to dryness *in vacuo*. The residue was dissolved in aqueous Na_2CO_3 (2·0 g. of Na_2CO_3, $10H_2O$) and the alkaline solution was distilled in a gentle stream of N_2. The effluent vapours were scrubbed through aqueous 2N-HCl and on evaporating the distillate to dryness there remained 0·399 g. of colourless methylamine hydrochloride, m.p. 225°, corresponding to 90·4% of 1 mole/mole of cyclopenin. The methylamine hydrochloride was purified for analysis by crystallization from ethanol and was obtained as colourless, glistening plates, m.p. 229° alone or in admixture with an authentic specimen. (Found: C, 18·0; H, 8·9; N, 21·0; Cl, 52·6. Calc. for CH_6NCl: C, 17·8; H, 9·0; N, 20·7; Cl, 52·5%.) The identity of the methylamine was confirmed by benzoylating it with benzoyl chloride in a Schotten Baumann reaction. The crude product so obtained was purified by sublimation in high vacuum at 60° followed by crystallization from light petroleum (b.p. 40–60°), giving *N*-methylbenzamide as long, fine, colourless needles, m.p. 81° not depressed on admixture with an authentic specimen, m.p. 82°. (Found: C, 71·4; H, 6·55. Calc. for C_8H_9ON: C, 71·1; H, 6·7%.)

Viridicatin

Five different strains of *P. cyclopium* isolated in this Department, were used for the preparation of viridicatin. Their history is summarized in Table 2.

Table 2. *History of cultures of* Penicillium cyclopium *used*

L.S.H.T.M. catalogue no.	Date of isolation	Source
525	October 1951	Garden soil ex Carron, Co. Clare, Eire
578	October 1951	Woodland soil ex Forest of Dean, Glos.
716	June 1952	Soil ex sand dunes, Perranporth, Cornwall
747	July 1952	Woodland soil ex copse, Watton at Stone, near Hertford
754	July 1952	Cultivated soil ex potato field, Watton at Stone, near Hertford

We are indebted to Mr G. Smith for the following description of these cultures. 'In culture on Czapek-agar all five strains are very similar being indeed virtually indistinguishable. Colonies are thick velvety, granular in the younger areas, blue-green turning gradually to grey-green with a broad white edge and regular outline; drops colourless; reverse pale peach colour to pale tan. Conidiophores delicately roughened, fasciculate; penicilli compact, mostly 3-verticillate; conidia globose or subglobose, smooth, mostly 3·5 μ. in diameter. The colony colours and the rough conidiophores united in fascicles leave no doubt that all the five isolates belong to *P. cyclopium* Westling.'

Isolation and purification of viridicatin

Batches, usually of one hundred 1 l. conical flasks, each containing 350 ml. of Czapek-Dox solution, were sterilized and sown with a spore suspension of the chosen strain of *P. cyclopium* grown on Czapek-agar slopes. The flasks were incubated at 24° for the period of time shown in Table 3. After 4–5 days the surface of the liquid was covered with a blue-green mycelial mat which began to curl slightly after 2 weeks. The reverse of the mycelium was very often pitted with long white needles of palitantin and had brown to dark brown patches. The pigment partly diffused into the culture fluid giving it a brownish colour when the flasks were harvested.

Antifungal tests on the culture filtrates from strains 525 and 578 were carried out by the method of Brian & Hemming (1945) using spores of *Botrytis allii* Munn. as the test organism and adjusting the pH of the germination medium to 4·0 with H_3PO_4. Both strains showed antifungal activity, and the details for strain 525 are as follows: after 7 days, no germination of spores at a dilution of the culture filtrate of 1:32; after 9, 16, 18 and 22 days, no germination at a dilution of 1:64.

After 22 days' incubation, forty-three flasks of strain 525 were harvested and the culture filtrate was extracted twice with half its volume of ether. Removal of the solvent gave 12·5 g. of a brownish, largely crystalline solid consisting mainly of palitantin (Birkinshaw & Raistrick, 1936; Birkinshaw, 1952) but giving a purplish red ferric reaction owing to the presence of frequentin (Curtis, Hemming & Smith, 1951; Curtis & Duncanson, 1952). The brown solid was fractionated as follows. 5 g. were triturated with ether (150 ml.) to remove readily soluble impurities. The residual

Table 3. *Cultural characteristics and yields of metabolic products of strains of* Penicillium cyclopium

For details see text.

L.S.H.T.M. catalogue no.	Incubation period (days)	pH of culture filtrate	Residual glucose (by polarimeter) (%)	Weight of mycelium (g.)	Weight of viridicatin (g.)	Weight of palitantin (g.)
525*	22	5·5	0·26	107	0·6	10·2
578	24	6·4	0·10	200	3·1	1·2
716	24	6·2	0·07	212	3·2	4·1
747	24	5·2	0·64	207	6·5	9·2
747	26	5·6	0·30	225	3·7	9·1
747	16	4·8	0·87	222	3·8	14·1
747	21	5·5	0·35	225	1·0	7·7
747	21	5·2	0·50	230	1·7	9·4
747	21	5·4	0·60	247	2·0	9·0
754	21	6·0	0·48	230	1·9	11·5
754	18	5·2	1·01	225	3·6	22·9

* This batch consisted of only forty-three flasks, all other batches of 100 flasks.

solid was dissolved in $CHCl_3$ (200 ml.) and the $CHCl_3$ solution was shaken with 2 N-Na_2CO_3 (50 ml.) to extract frequentin.

Removal of the $CHCl_3$ gave palitantin (4·08 g. ≡ 10·2 g. for forty-three flasks) which was purified by crystallization from ethyl acetate giving colourless needles m.p. 164–165°, not depressed on admixture with an authentic specimen from *P. palitans*. (Found: C, 66·2; H, 8·6. Calc. for $C_{14}H_{22}O_4$: C, 66·1; H, 8·7%.)

The Na_2CO_3 extract was acidified with HCl. A brown oil separated and crystallized overnight at 0°. It was purified by crystallization first from water (charcoal) (0·63 g. ≡ 1·6 g. for forty-three flasks, m.p. 128–132°) and then from benzene giving frequentin as colourless plates, m.p. 134° not depressed on admixture with an authentic specimen from *P. frequentans* and giving the same purple-red ferric colour. (Found: C, 66·7; H, 8·1. Calc. for $C_{14}H_{20}O_4$: C, 66·6; H, 8·0%.)

On similar treatment, the culture filtrate from forty-three flasks of strain 578, grown for 22 days, gave 11·2 g. of crude ether extract from which palitantin and frequentin were isolated.

The culture filtrates from strains 578, 716, 747 and 754 were extracted with $CHCl_3$ for the recovery of palitantin. We are indebted to Dr A. Kamal for the experimental data concerning this substance given in Table 3, column 7.

The mycelium was washed with a little water, drained, pressed and dried *in vacuo* at 40–50°. The dried mycelium was ground and extracted in a Soxhlet with light petroleum (b.p. 40–60°) for 8 hr. Removal of the solvent from this extract gave an oily, partly crystalline residue containing fat, palitantin and traces of viridicatin. The light-petroleum-extracted mycelium was dried and re-extracted with ether until the ether extract no longer gave a green ferric reaction. Removal of the ether gave a brownish crystalline residue of crude viridicatin, melting usually in the range 250–258°, which was purified by repeated fractional crystallization from methanol. Viridicatin was thus obtained as colourless needles, m.p. 268°. (Found: C, 75·5; H, 4·7; N, 6·4. Calc. for $C_{15}H_{11}O_2N$: C, 75·9; H, 4·7; N, 5·9%.)

General properties of viridicatin

Viridicatin crystallizes in colourless, shining needles which show a definite violet fluorescence on exposure to ultraviolet light. No fluorescence is shown by cyclopenin under the same conditions. Viridicatin melts at 268° without decomposition and the melt resets on cooling at 250° and remelts at 268°. A sublimate of colourless well defined and characteristic prisms is formed in the m.p. tube. It readily sublimes unchanged in high vacuum at 160–170°. It crystallizes readily from ethanol (0·78 g. in 60 ml. ethanol). Its ethanolic solution (0·25%) showed no optical rotation in a 4 dm. tube. It does not dissolve in aqueous $NaHCO_3$ but dissolves readily in cold aqueous 2 N-KOH and is precipitated in crystalline clusters on saturating this solution with CO_2. It dissolves with some difficulty in cold conc. HCl but readily in glacial acetic acid and is precipitated in fine, colourless needles on diluting either solution with water. It gives no colour with either hot or cold conc. H_2SO_4, but on addition of $NaNO_2$ to the latter solution, a deep brownish red colour is produced. Addition of Br_2 water to a solution of viridicatin in dilute aqueous acetic acid gives no precipitate (cf. cyclopenin). The most characteristic colour reaction of viridicatin is the intense emerald-green colour given immediately (cf. cyclopenin) on addition of small amounts of $FeCl_3$ to its ethanolic solution. This colour is completely discharged on addition of conc. HCl and changes to a deep orange-red on addition of a few drops of aqueous N-NaOH, the original green colour being restored in each case on adjustment to a slightly acid pH.

Derivatives of viridicatin

Monoacetylviridicatin. A solution of viridicatin (0·1 g.) in acetic anhydride (0·6 ml.) was refluxed for 15 min., cooled, poured into water (4 ml.) and boiled for 1 min. The colourless crystals which separated on cooling were collected and recrystallized from aqueous ethanol giving *monoacetylviridicatin* (0·05 g.) as short colourless prisms, m.p. 200–201°. (Found: C, 72·6; H, 4·7; N, 5·0, 5·3. $C_{17}H_{13}O_3N$ requires C, 73·1; H, 4·7; N, 5·0%.) The acetate decomposes slowly on standing, but a freshly prepared specimen gives no green ferric colour in ethanol solution nor is it soluble in aqueous Na_2CO_3.

3:5-*Dinitrobenzoic acid salt.* Pure 3:5-dinitrobenzoic acid (0·2 g.) was added to a hot saturated solution of viridicatin (0·1 g.) in glacial acetic acid. The bright yellow solution, on cooling, deposited rosettes of yellow prisms of the *3:5-dinitrobenzoic acid salt of viridicatin* (0·2 g.), m.p. 228–230° raised to a constant m.p. of 238° by recrystallization from glacial acetic acid. (Found: C, 58·4; H, 3·4; N, 9·75. $C_{15}H_{11}O_2N.C_7H_4O_6N_2$ requires C, 58·8; H, 3·4; N, 9·4%.)

OO-*Dimethylviridicatin*. A mixture of viridicatin (1 g.), methanol (25 ml.) and a large excess of Ag_2O and methyl iodide was refluxed for 4 hr., cooled, filtered and the residue washed with methanol. The clear yellow filtrate which no longer gave a ferric reaction, was decolorized with a little charcoal and filtered. On removal of the solvents from the filtrate there remained a white residue (1·0 g.), m.p. 60–140°, consisting of a mixture of *OO*-dimethyl and *ON*-dimethylviridicatin which was fractionated from light petroleum (b.p. 40–60°). Two crude fractions were obtained. (*a*) 0·15 g., m.p. 160–180°, insoluble in light petroleum, contained the *ON*-dimethyl ether which is more conveniently prepared as described below. (*b*) 0·7 g., m.p. 70–110°, soluble in light petroleum, was purified by fractional crystallization from ethanol, giving OO-*dimethylviridicatin* as colourless glistening plates, m.p. 86–87°. (Found: C, 76·5; H, 5·7; N, 5·5; OMe, 23·7. $C_{17}H_{15}O_2N$ requires C, 76·95; H, 5·7; N, 5·3; 2OMe, 23·4%.)

ON-*Dimethylviridicatin*. A mixture of viridicatin (1 g.), methanol (50 ml.) and dimethyl sulphate (3 ml.) was refluxed, and NaOH pellets (total, 2 g.) were added from time to time to keep the reaction alkaline. After 1·5 hr. a green ferric reaction could no longer be detected. Water (70 ml.) was added to the hot solution from which, on cooling, the crude methyl ether separated (0·95 g., m.p. 184–190°). Recrystallization from ethanol gave ON-*dimethylviridicatin* as colourless glossy plates, m.p. 197–198°. (Found: C, 76·65; H, 5·7; N, 5·2; OMe, 11·7, 11·25. $C_{17}H_{15}O_2N$ requires C, 76·95; H, 5·7; N, 5·3; 1 OMe, 11·7%. In the Zeisel methoxyl estimation the temperature was not allowed to rise above 130–135° to avoid demethylation of the N.Me group.)

Degradation of viridicatin

(*a*) *By alkaline oxidation: Isolation of o-aminobenzophenone* (I) *and oxalic acid*. CO_2-free O_2 was bubbled through a solution, held at room temp., of viridicatin (0·5 g.) in 10% ethanolic KOH (50 ml.). After a few minutes the clear colourless solution became yellow and the depth of colour increased with time. The reaction was stopped after 7–8 hr., when the solution no longer gave any green ferric colour, and the pH was adjusted to about 5 with aqueous 2N-HCl. Ethanol was removed *in vacuo*. On cooling, yellow crystals of *o*-aminobenzophenone (I) separated and were collected together with a little more extracted with ether from the mother liquors. Combined yield, 0·42 g., m.p. 95–100°. The product was purified by repeated sublimation in high vacuum at 70–80° giving pure *o*-aminobenzophenone as yellow plates, m.p. 106–107°, not depressed on admixture with an authentic synthetic specimen of the same m.p. (Scheifele & DeTar, 1952). (Found: C, 78·6; H, 5·6; N, 7·3. Calc. for $C_{13}H_{11}ON$: C, 79·2; H, 5·6; N, 7·1%.) The monoacetyl derivative of the degradation product, prepared in the usual way, crystallized from aqueous methanol as colourless tablets, m.p. 88–89° alone or in admixture with an authentic synthetic specimen of the same m.p. (Found: C, 75·2; H, 5·5; N, 5·9. Calc. for $C_{15}H_{13}O_2N$: C, 75·3; H, 5·5; N, 5·9%.) The hydrochloride, long colourless needles from conc. HCl, m.p. 175° (decomp.), and the mono-2:4-dinitrophenylhydrazone, dark red, diamond-shaped crystals from glacial acetic acid, m.p. 261–262° (decomp.), were also prepared.

In a second experiment viridicatin (0·48 g.) was oxidized as described above. The oxidation solution was adjusted to pH 6·5 with N acetic acid (140 ml.). Ethanol was removed *in vacuo* and the *o*-aminobenzophenone which separated was collected by filtration and washed well with water. The combined filtrate and washings were acidified with glacial acetic acid (1 ml.), and 0·5N-$CaCl_2$ (22 ml.) was added. Calcium oxalate which separated as a white ppt. was collected next day and dried at 110° (0·264 g.; calc. for 1 mole $(COO)_2Ca$, H_2O = 0·297 g.). The ppt. was then suspended in 2N-H_2SO_4 (100 ml.) and extracted continuously with ether for 3 days. The ether extract was dried (Na_2SO_4) and the solvent removed. The residue was twice sublimed in high vacuum at 80–90° and the sublimate (0·1 g.) was equilibrated with water vapour to give oxalic acid dihydrate, m.p. 101°. (Found: C, 19·5; H, 5·1. Calc. for $(COOH)_2$, $2H_2O$: C, 19·1; H, 4·8%.)

(*b*) *By* $KMnO_4$ *oxidation in acetone: Isolation and synthesis of oxalyl-o-aminobenzophenone* (III). $KMnO_4$ (6·0 g.) was added in two portions to a solution of viridicatin (1 g.) in acetone (200 ml.), and the mixture was refluxed for 4 hr. The acetone solution was then nearly colourless and was separated by filtration and discarded. The dark MnO_2 precipitate was suspended in 2N-H_2SO_4 (200 ml.) and SO_2 was passed until a clear solution was obtained. Pale yellow needles separated on cooling and were collected (0·95 g., m.p. 160–175°). This product was crystallized several times from $CHCl_3$ (charcoal) and *oxalyl-o-aminobenzophenone* (III) was thus obtained in clusters of fine colourless needles, m.p. 184° (decomp.). (Found: C, 66·3; H, 4·2; N, 5·0; equiv. by potentiometric titration, 270. $C_{15}H_{11}O_4N$ requires C, 66·9; H, 4·1; N, 5·2%; equiv. titrating as a monobasic acid, 269.) (III) (0·265 g.; m.p. and mixed m.p. 184°) was also obtained by oxidizing viridicatin (0·5 g.) at 100° with H_2O_2 (100 vol.; 5 ml.) in glacial acetic acid (20 ml.).

Freshly distilled ethoxalyl chloride (0·3 ml.; Fourneau & Sabetay, 1928) was added to a solution of *o*-aminobenzophenone (0·1 g.) in pyridine (0·5 ml.). When the initial vigorous reaction had subsided the mixture was warmed on the water bath for 15 min., cooled and poured into water (10 ml.). A colourless oil, which quickly solidified, separated and was collected (0·137 g., m.p. 70–115°). Crystallization from ethanol gave *ethoxalyl-o-aminobenzophenone* (II) as colourless irregular plates, m.p. 123–124°. (Found: C, 68·6; H, 5·2; N, 4·85. $C_{17}H_{15}O_4N$ requires C, 68·7; H, 5·1; N, 4·7%.)

The pure ester (II; 0·125 g.) was dissolved in aqueous N-NaOH (2 ml.) and held at room temp. for 20 min. A little of the ester was completely hydrolysed to *o*-aminobenzophenone (I), but, after acidification with 2N-HCl (1 ml.), oxalyl-*o*-aminobenzophenone (III) separated and was collected in good yield as a yellowish product (0·11 g.; overall yield, 81%), m.p. 172–179° (decomp.), raised to 184° (decomp.) on crystallization from $CHCl_3$ and not depressed on admixture with the $KMnO_4$ oxidation product from viridicatin. (Found: C, 66·7; H, 3·9; N, 5·65. $C_{15}H_{11}O_4N$ requires C, 66·9; H, 4·1; N, 5·2%.)

Synthesis of viridicatin

o-Aminobenzophenone (I; 5 g.), m.p. 105°, was dissolved in warm (temp. not above 100°) molten chloroacetic anhydride (6·5 g.). The orange solution was kept below 100° for 1 min. and was then cooled to room temp. The reaction mixture quickly crystallized and finally solidified. The product was ground in ice cold water (50 ml.), filtered, washed with water and dried (5·48 g., m.p. 92–96°). Two crystallizations of the product from methanol gave *chloro-*

acetyl-o-aminobenzophenone (IV) as colourless long needles, m.p. 107–108°. (Found: C, 65·8; H, 4·3; N, 5·8; Cl, 13·2. $C_{15}H_{12}O_2NCl$ requires C, 65·8; H, 4·4; N, 5·1; Cl, 13·0%.)

Cyclization of the chloroacetyl derivative was effected by dissolving it (5·36 g.) in hot ethanol (50 ml.) and diluting with water (140 ml.). When the solution became clear again, NaOH (1·5 g.) in water (10 ml.) was added, and a yellow colour developed owing to partial hydrolysis. The solution was refluxed for 30 min. and acidified with HCl while still hot. On cooling, colourless, chlorine-free, prismatic needles separated and were collected (4·3 g., 72% overall yield, m.p. 240–242°). Crystallization from ethanol gave synthetic viridicatin, m.p. 264–265°, not depressed on admixture with natural viridicatin from *P. cyclopium*. (Found: C, 75·7; H, 4·7; N, 6·1. Calc. for $C_{15}H_{11}O_2N$: C, 75·9; H, 4·7; N, 5·9%.) The two specimens also gave identical intense emerald-green ferric reactions. The *ON*-dimethyl derivative of synthetic viridicatin, prepared as described above, melted at 197–198° and gave no depression in m.p. on admixture with the *ON*-dimethyl derivative of natural viridicatin of the same m.p. (Found: C, 76·8; H, 5·7; N, 5·9. Calc. for $C_{17}H_{15}O_2N$: C, 76·95; H, 5·7; N, 5·3%.)

SUMMARY

1. A hitherto undescribed mould metabolic product, cyclopenin, has been isolated from culture filtrates of laboratory cultures of a strain of *Penicillium cyclopium* Westling.

2. Cyclopenin, $C_{17}H_{14}O_3N_2$, forms colourless tablets, m.p. 207°, $[\alpha]_{5461}^{20} - 306°$ in ethanol.

3. Cyclopenin is readily decomposed by dilute mineral acids giving one mole each of carbon dioxide, methylamine and viridicatin, $C_{15}H_{11}O_2N$, which is optically inactive.

4. Viridicatin, previously isolated from *P. viridicatum* Westling, by Cunningham & Freeman (1953), has now been prepared from five different strains of *P. cyclopium* but appears to be absent from the strain of this species which gives cyclopenin.

5. Viridicatin is oxidized in alkaline solution by gaseous oxygen to one mole each of *o*-aminobenzophenone and oxalic acid.

6. Viridicatin has been synthesized and has been shown to be 2:3-dihydroxy-4-phenylquinoline or its keto tautomer.

7. The five strains of *P. cyclopium* which gave viridicatin also gave good yields of palitantin and two of them gave the anti-fungal substance, frequentin.

8. Two possible, closely related, structures are suggested for cyclopenin.

We wish to thank Sir Robert Robinson, O.M., F.R.S., for his friendly advice on the structure of cyclopenin.

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The Purification of Alkaline Phosphatases of Animal Tissues

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Despite the fact that much of the considerable literature concerning alkaline phosphatases is devoted to methods for purification, no procedure for obtaining a homogeneous preparation from animal tissues has so far been described. This may possibly be due to the fact that the cytoplasmic alkaline phosphatases are intimately associated with insoluble cellular granules. Special procedures must therefore be used to obtain these enzymes in true solution in order to permit of purification.

The difficulty of extracting alkaline phosphatases from tissue 'homogenates' was recognized by early investigators (see Ehrensvärd, 1933). It was found, however, that various forms of proteolysis, such as controlled autolysis (Albers & Albers, 1935) and

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digestion with trypsin (Ehrensvärd, 1933; Schmidt & Thannhauser, 1943) enabled release of some of the enzyme into true solution. This was confirmed by Kabat (1941) who showed that alkaline phosphatase of mouse kidney is sedimented by centrifuging at 27 000 rev./min. for 1 hr. while, after prolonged autolysis, most of the enzyme remained in the supernatant.

As will be indicated in the Discussion, however, there are numerous objections to the use of proteolytic procedures for separating enzymes from particulate material in order to permit of their purification. Therefore, when a purified preparation of alkaline phosphatase of calf intestinal mucosa was required for studies of the relationship between the hydrolytic and transferase activities (see Morton, 1953*a*), other methods of separating the enzyme from the insoluble particles were sought. It was confirmed that phosphatase of calf intestinal mucosa (as well as of other tissues) was associated with insoluble cellular particles. By treatment with butanol the enzyme was quantitatively released into true solution. Subsequent fractionation yielded a preparation which was more active than any previously described and the evidence available suggested that it was essentially homogeneous. The principles of the purification procedure have been briefly reported (Morton, 1950).

MATERIALS AND METHODS

Enzymic activities

Alkaline phosphatase. Both the unit of activity and the specific activity have been defined in a previous paper (Morton, 1953*b*). Activities were determined essentially as therein described, using 4·8 ml. of buffered substrate and 0·2 ml. of suitably diluted enzyme. Final concentrations of the reactants were: sodium β-glycerophosphate (0·02 M), either veronal–HCl (0·04 M) or ethanolamine–HCl buffer (0·02 M), pH 9·5 at 38°, and magnesium acetate (0·001 or 0·01 M). The lower concentration of Mg^{2+} was used in testing the crude enzyme preparation, since higher concentrations are inhibitory to these (Abul-Fadl & King, 1949*a*; Morton, 1952). The amount of enzyme was adjusted so that hydrolysis was never more than 5% of the total substrate, and was usually less than 1%. Under these conditions, the rate of hydrolysis is proportional to enzyme concentration.

Other enzymes. Diphosphopyridine nucleotide–cytochrome *c* reductase, diaphorase, and succinic dehydrogenase activities were determined essentially as previously described (Morton, 1954), except that reduction of dyes was estimated visually, the tests being qualitative only.

Chemical estimations

Inorganic phosphate was determined by the method of Fiske & Subbarow (1925).

Nitrogen was estimated by a micro-Kjeldahl procedure or by a modification of the method described by Johnson (1941) using Nessler reagent. For rapidly following purification procedures, the maximum absorption between 270 and 280 mμ. (usually 278 mμ.), as determined in a Beckman spectrophotometer, was used as a measure of protein concentration. The absorption values were converted to protein nitrogen by frequent calibration using the procedures previously mentioned.

Dry weights were determined after freezing and drying *in vacuo* over conc. H_2SO_4 for 48 hr. at room temp. (18°).

Lipid was estimated by the loss in dry weight after repeated extraction of the trichloroacetic acid precipitate with ether–ethanol (1:3) at room temp.

EXPERIMENTAL AND RESULTS

Localization of alkaline phosphatase of calf intestinal mucosa

The intestine of a 3-day-old calf was collected as soon as possible after killing. The convolutions of the intestine were removed by cutting away the mesenteric tissue with scissors and the small intestine was thoroughly rinsed by forcing a strong jet of running tap water through it. Portions of the intestine, about 2 ft. in length, were laid flat on an enamelled tray and the mucosa forced out by scraping the intestine with the edge of a plastic spatula. The mucosa was transferred to a beaker and cooled to 0° in an ice bath.

About 20 g. of mucosa were suspended in 200 ml. of 0·25 M sucrose (at 0°) and gently stirred with a mechanical stirrer for 15 min. The sucrose suspension was then fractionated by differential centrifuging as shown in Fig. 1.

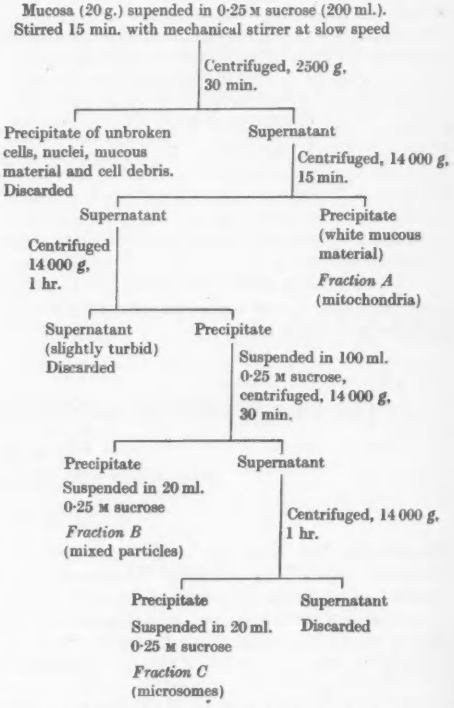
Mucosa (20 g.) supended in 0·25 M sucrose (200 ml.).
Stirred 15 min. with mechanical stirrer at slow speed
Centrifuged, 2500 g, 30 min.
Precipitate of unbroken cells, nuclei, mucous material and cell debris. Discarded
Supernatant
Centrifuged, 14 000 g, 15 min.
Supernatant
Precipitate (white mucous material)
Fraction A (mitochondria)
Centrifuged 14 000 g, 1 hr.
Supernatant (slightly turbid) Discarded
Precipitate
Suspended in 100 ml. 0·25 M sucrose, centrifuged, 14 000 g, 30 min.
Precipitate
Suspended in 20 ml. 0·25 M sucrose
Fraction B (mixed particles)
Supernatant
Centrifuged, 14 000 g, 1 hr.
Precipitate
Suspended in 20 ml. 0·25 M sucrose
Fraction C (microsomes)
Supernatant
Discarded

Fig. 1. Fractionation of intestinal mucosa. All procedures carried out at 0–2°.

Because of the limited centrifugal force (maximum about 14000 *g*) which was available, many of the smaller particles could not be completely sedimented, and the final supernatants were either cloudy or opalescent. Therefore, a study of the quantitative distribution of alkaline phosphatase did not seem justified, and instead the specific activities of the various fractions were determined. Qualitative tests were carried out for diaphorase, diphosphopyridine nucleotide–cytochrome *c* reductase and succinic dehydrogenase activities. The results are shown in Table 1.

A portion of each of the fractions *A*, *B* and *C* was fixed by addition of neutral formaldehyde (40%) to give a final concentration of 2% in the sucrose suspension. The particles were then sedimented by centrifuging (14000 *g*, 1 hr., 0–2°), washed twice with 40 ml. water and finally suspended in water (5 ml.). Various dilutions of each fraction were then transferred to cellulose nitrate films, the specimens dried *in vacuo*, and shadowed with gold–palladium at an angle of approx. 20°. They were examined in a Siemens electron-microscope.

The particles of fraction *A* were of irregular size and shape and were largely aggregated. Among the fields examined there appeared to be few particles less than 200 mμ. in diameter. The various dilutions of fraction *B* showed particles which were mostly of about 100–300 mμ. in diameter, although some were less than 100 mμ. There was some aggregation. Fraction *C* contained many particles which appeared to be spherical. They ranged from about 200 mμ. to less than 20 mμ. in diameter with a mode of about 80 mμ.

Localization of alkaline phosphatase of cow mammary gland

A mammary gland from a lactating cow, obtained from the slaughter house within 2 hr. of death, was cooled in ice. Two sections of tissue from different quarters were minced and washed twice in 0·25M sucrose (at 0°) to remove milk. About 100 g. of mince was then disintegrated for 1 min. in a Waring Blendor in 500 ml. of sucrose (0·25M). The 'homogenate' was centrifuged at 2500 *g* for 20 min., at 0° and the precipitate discarded. The supernatant was centrifuged at 14000 *g* for 15 min. and the precipitate (fraction M_1) resuspended in 5 ml. sucrose (0·25M). The supernatant was then centrifuged at 20000 *g* for 1 hr. in the high-speed attachment of an International centrifuge and the precipitate (fraction M_2) resuspended in 5 ml. sucrose. The final supernatant (fraction M_3) was still slightly turbid.

The specific alkaline phosphatase activities of the fractions are shown in Table 2.

Table 1. *Properties of fractions from calf intestinal mucosa*

Cytochromes observed in a thick smear of mucosa or precipitate, reduced with $Na_2S_2O_4$, using a low-dispersion micro-spectroscope. Enzymes estimated as described under Methods. + Indicates qualitative test positive; − indicates negative.

		Fractions (see Fig. 1)		
	Whole 'homogenate' in 0·25M sucrose	*A* (mitochondria)	*B* (mixed particles)	*C* (microsomes)
Approximate size of particles	Not determined	300 mμ.	300–100 mμ.	200–20 mμ.
Appearance of particles	Not examined	Irregular clumps	Some large particles and aggregates	Spheroidal isolated particles
Lipid (%)	Not determined	Not determined	Not determined	29
Cytochrome components	*a*, *c*	Not determined	Not detected	Not detected
Alkaline phosphatase (units/mg. N)	72	50	240	640
Diphosphopyridine nucleotide–cytochrome *c* reductase	+	Not determined	+	+
Diaphorase	+	Not determined	+	+
Succinic dehydrogenase	+	+	+	−

Table 2. *Properties of fractions from cow mammary gland*

For explanation of symbols, see Table 1. Xanthine oxidase tested as a dehydrogenase (see Methods).

		Fraction, and conditions of sedimentation		
	Whole 'homogenate' in 0·25M sucrose	M_1 14000 *g*, 15 min. (mitochondria)	M_2 20000 *g*, 1 hr. (microsomes)	M_3 Remaining supernatant
Appearance of precipitates	Salmon-pink in colour	Tan	Brown	Opalescent
Cytochrome components	*a*, *c*	*a*, *b*, *c*	Not detectable	Not detectable
Alkaline phosphatase (units/mg. N)	11	20	65	4
Xanthine oxidase	+	+	+	+
Diaphorase	+	+	+	−
Succinic dehydrogenase	+	+	+	−

Separation of alkaline phosphatase activity from particulate components of animal tissues

In a previous paper (Morton, 1953*b*) it was shown that treatment of milk or milk products with *n*- or *iso*-butanol released the alkaline phosphatase into true solution from the lipoprotein particles. The same method was therefore examined with dispersions of several different tissues from the rat and rabbit. The tissue was ground in a mortar with an equal weight of fine acid-washed sand, and with twice its weight of 0·05 M veronal–HCl buffer at pH 8·0. A small sample of this dispersion was removed. A volume of *n*-butanol equal to the weight of tissue was then added, the material stirred at 35° for 15 min. and separated into distinct phases by centrifuging (2500 *g*, 45 min., at room temp.). The volume of the clear aqueous phase was measured, and a sample removed for alkaline phosphatase determination. The aqueous extracts contained over 80% of the total alkaline phosphatase of the untreated dispersions, while complete recovery of the enzyme could be obtained by re-extraction with butanol saturated 0·1 M-$NaHCO_3$. Moreover, the enzyme was in true solution, since neither centrifuging in the high-speed attachment of the International centrifuge (20000 *g*, 1 hr.), nor slow filtration through a thick bed of Hyflo Supercel (Johns Mansville and Co.) reduced the enzymic activity. In some cases, especially when intestinal dispersions were used, the aqueous layer remaining after butanol treatment of the tissue homogenate was slightly turbid even after prolonged centrifuging at 2500 *g*. This turbidity appeared to be due to mucous material which, however, was not associated with the enzyme, since slow filtration through Hyflo Supercel yielded a clear aqueous extract containing the total enzyme. The results of this survey of the effectiveness of butanol for release of the enzyme from various tissues are summarized in Table 3.

The aqueous extracts obtained after butanol treatment of animal tissues may be dialysed free of butanol and then frozen and dried *in vacuo* with very little loss of phosphatase activity. Moreover, the butanol-saturated aqueous material may be stored at 0–2° for considerable periods (experimental observations continued for 8 months in one case) without visible bacterial contamination and with only slight decline in activity.

Purification of alkaline phosphatase of calf intestinal mucosa

Calf intestines were collected from the slaughter house as soon as possible after killing, and the mesenteric membranes removed. The mucosa was then obtained as described previously, transferred to a stainless-steel beaker and immediately cooled to 0–2° in a refrigerated bath. About 3 l. of mucosa were obtained from the intestines of forty calves.

Table 3. *Alkaline phosphatase activities of aqueous extracts of animal tissues treated with butanol*

The tissue 'homogenates' (at pH 7·4) were treated with excess *n*-butanol at 35° for 15 min., centrifuged, and the aqueous layer was removed and tested (see above).

Tissue	Activity of extract (Units/ml.)	Activity of extract (Units/mg. N)	Recovery of enzyme (%)
Rat kidney	48	35	87
Rabbit kidney	118	60	—
Rabbit liver	59	26	82
Rat intestine	95	30	94
Rabbit heart	17	11	—
Rabbit skeletal muscle	5	—	—

The 'homogenate' of mucosa rapidly undergoes autolysis, which releases some of the phosphatase from the particulate material. This is to be avoided, not only because it results in loss of enzyme, but also because it is desirable to prevent contamination of the enzyme by products of proteolysis. It is therefore necessary to proceed with the preparation as quickly as possible after collection of the mucosa.

Stage 1. The mucosa was dispersed in 3 vol. of water (at 0°) and adjusted to pH 7·5 by slow addition of N-NaOH. After vigorous mechanical stirring for about 30 min. at 0°, the 'homogenate' was centrifuged (1800 *g*, 40 min.) in aluminium cups (1300 ml. capacity) in an International Serum Centrifuge (total capacity 13 l.). The supernatant was poured off and filtered through a layer of well-washed cotton wool on a Büchner funnel in order to remove loose fat and other insoluble aggregates. The filtrate was immediately cooled to 0° in a stainless-steel container held in a cold bath at −10°. The precipitate, which contained no more than 10% of the phosphatase of the 'homogenate', was discarded.

The initial disintegration of the mucosa was obtained using a 4-blade stainless-steel stirrer, driven at high speed by a 0·05 h.p. motor. Alternatively, the mucosa may be disrupted in a Waring-type blender for about 2 min., using 3 vol. distilled water.

Stage 2. The filtrate at 0–2° was adjusted to pH 5·0 by addition of 2 M acetate buffer, pH 4·0, held for 45 min. at 0° and then centrifuged (1800 *g*, 40 min. at room temp.). The white precipitate was then thoroughly dispersed in 8 l. of 0·15 M-NaCl. The suspension was adjusted to pH 7·5 with 0·5 M-Na_2CO_3, cooled to 0° and stirred gently at 0–2° overnight. The enzyme was then reprecipitated at pH 5·0 as before, and similarly collected by centrifuging. At this stage the supernatant was clear and almost free of soluble protein. The precipitate was then washed in the cups by rubbing up into 8 l. of distilled water (at 0°) and immediately recentrifuged as previously. By this means the ionic strength of the material was lowered, and the last traces of soluble protein were removed.

This precipitate was again dispersed in distilled water at 0° and made up to 5 l. The white dispersion (now at pH 5·5) was at this stage free of mucous material and of low viscosity, while all traces of blood, pigments and soluble proteins had been removed.

As indicated in the Discussion it is preferable to sediment the particles carrying alkaline phosphatase by high-speed centrifuging at neutral pH, rather than by precipitating as described above. Therefore, where suitable equipment is available for centrifuging relatively large volumes at high speeds, the following modification (stage 2*a*) is recommended.

Stage 2*a*. The filtrate from stage 1 is centrifuged at 10000 *g* for 30 min. at 0–4°. The supernatant is then centrifuged at 40000–60000 *g* for 1–2 hr. The precipitate is resuspended in 5 vol. of 0·15 M-NaCl using a Waring-type blender (1 min.), the suspension readjusted to pH 7·2 with 0·2 M-$NaHCO_3$, and gently stirred overnight at 0–2°. The particles are again sedimented (40000–60000 *g*, 1 hr.). The precipitate is suspended in 5 vol. of distilled water, the suspension stirred for 1 hr. at 0–2°, and finally recentrifuged as before. The precipitate is suspended in 3 vol. of distilled water and then treated as described under stage 3.

Stage 3. About 2 l. of *n*-butanol were slowly added over a period of 15 min. with vigorous mechanical stirring. The material was then heated to 38° for 5 min. and immediately centrifuged in 1 l. glass cups (1800 *g*, 30 min.). The clear, colourless aqueous layer was removed by suction and filtered through a thick layer of Hyflo Supercel over no. 1 Whatman paper on a 1 l. Büchner funnel. The filtrate was then adjusted to pH 8·5, held at 0° overnight and again filtered through Hyflo Supercel to remove a small precipitate.

Stage 4. The filtrate was adjusted to pH 6·4, cooled to −5° and the enzyme directly precipitated at −5° by addition of ether (10%, v/v) and acetone (60%, v/v). After settling overnight at −7°, the supernatant was removed by suction, and the precipitate collected by centrifuging at −5°.

It is often convenient to dry the precipitate at this stage and to accumulate material from several batches of mucosa before proceeding further with purification. For this purpose, the precipitate is washed twice with dry acetone at −15° followed with dry anaesthetic ether at −15°, and then dried, initially in a stream of N_2 and finally *in vacuo* over H_2SO_4 and $CaCl_2$. The dry powder may be stored over NaOH and $CaCl_2$ *in vacuo* at 0°. It retains activity for at least 12 months. For further purification it is treated exactly as the undried precipitate.

Usually, drying was omitted and the 60% (v/v) acetone precipitate was dissolved in a minimum of 0·05 M veronal–HCl buffer at pH 6·4 and dialysed against 0·015 M magnesium acetate, at pH 6·4. Insoluble material was removed by centrifuging (20000 *g*, 1 hr., at 2°).

Stage 5. Following addition of ether (10%, v/v) the enzyme was precipitated at −5° with acetone between 35 and 48% (v/v). This fraction was dissolved in 0·05 M veronal–HCl buffer at pH 6·4 and dialysed gainst glass-distilled water (at 0°) until free of organic solvent.

Stage 6. The solution was adjusted to pH 4·9 with 0·05 N acetic acid, and heated rapidly to 48° for 2 min. A slight opalescence appeared. Magnesium acetate (0·5 M) was added to give a final concentration of 0·015 M, the solution adjusted to pH 6·4 and the enzyme precipitated at −5° with acetone between 40 and 50% (v/v). The slight precipitate at 40% (v/v) acetone was discarded. The precipitate between 40 and 50% (v/v) acetone was dissolved in buffer and dialysed free of organic solvent as for stage 5.

Stage 7. The solution was adjusted to pH 5·5 with 0·5 N acetic acid, and successive 20 mg. lots of washed charcoal, prepared as previously described (Morton, 1953*c*) were added slowly after mixing to a thick slurry in distilled water. Each precipitate was removed by centrifuging (5000 *g*, 15 min., at 2°) and the procedure repeated until about 30% of the enzymic activity was adsorbed. The solution was then centrifuged (14000 *g*, 30 min.), adjusted to pH 8·0 with 0·1 N-NaOH, and filtered on a Büchner funnel through a thin layer of magnesium carbonate over a no. 1 Whatman filter paper in order to remove colloidal charcoal.

Stage 8. The enzyme solution was adjusted to pH 6·4 with 0·1 N acetic acid and dialysed at 2° against 0·015 M magnesium acetate for 4 hr. with continuous stirring. The solution was cooled to 0°, ether added to 10% (v/v) and the enzyme precipitated at −5° with acetone between 40 and 48% (v/v). The precipitate was dissolved in glass-distilled water, and dialysed against glass-distilled water (at 0°) for 4 hr. with frequent changes of water.

A summary of the procedure and the results for preparation no. 6 are reported in Table 4. Preparation no. 6 gave a final product of specific activity of 83 500 units/mg. N (Q_p 579 000) compared with the initial supernatant of activity 97 units/mg. N, representing a purification on this basis of 860 times. Since much inert material was removed during the first centrifuging, the total purification compared to the activity of the original mucosa was well over 1000 times. On the basis of non-dialysable dry weight, the total purification was estimated to be about 1700 times. The final yield was 6% of the activity of the first supernatant.

The dialysed solution from stage 8 (Table 4) was frozen and dried *in vacuo* over H_2SO_4. The dried material was stored *in vacuo* over $CaCl_2$ at 0°. This preparation lost about 40% of its activity after storage for 12 months in the dry state. It is noteworthy that dilute solutions of pure alkaline phosphatase may decline considerably in activity when held at −15°, apparently due to irreversible protein denaturation.

During investigation of various purification procedures, alkaline phosphatase was prepared by adding *n*-butanol

Table 4. *Purification of alkaline phosphatase from calf intestinal mucosa*

Stage	Vol. (ml.)	Activity (units/ml.)	Total units ($\times 10^3$)	Total N (mg./ml.)	Specific activity (units/mg. N)	Yield (%)	Purification
1	12 000	205	2 460	2·12*	97	100	1
2	5 000	216	1 080	0·82	263	44	3
3	4 450	166	739	0·03	5 533	30	57
4	55	12 360	680	2·05	6 029	28	62
5	20	26 250	525	1·96	13 393	21	138
6	20	18 900	378	0·94	20 106	15	208
7	32	7 340	235	0·11	66 727	10	688
8	10	14 200	142	0·17	83 529	6	860

* Nitrogen estimated after dialysis, 24 hr. at 0°, against distilled water.

Summary of procedures

Stage	
1	Supernatant from 3 l. of mucosa in about 9 l. distilled water, pH 7·5, centrifuged 1800 *g*, 40 min.
2	Precipitate at pH 5·0, repeatedly washed and suspended in distilled water.
3	Filtered aqueous layer following butanol treatment, held pH 8·5, 14 hr. at 0° and refiltered.
4	Dialysed solution of precipitate obtained with ether (10%, v/v) and acetone (60%, v/v).
5	Dialysed solution of fraction obtained with ether (10%, v/v) and acetone (35–48%, v/v).
6	Dialysed solution after heating to 48°, 2 min. at pH 4·9 and fractionation with acetone (40–50%, v/v).
7	Filtrate following adsorption of inert protein on charcoal at pH 5·5.
8	Dialysed solution of fraction obtained with ether (10%, v/v) and acetone (40–48%, v/v).

Table 5. *Partial purification of alkaline phosphatase from calf intestinal mucosa*

Stage	Vol. (ml.)	Activity (units/ml.)	Total units ($\times 10^3$)	Total N (mg./ml.)	Specific activity (units/mg. N)	Yield (%)	Purification
1	2 000	600	1 200	7·70	78	100	1
2	1 750	555	971	0·77	721	81	9
3	1 900	465	885	0·28	1 661	74	21
4	105	7 850	824	2·92	2 688	69	35
5	30	17 200	516	2·70	6 370	43	82
6	20	22 000	440	1·90	11 579	37	149
7	18	16 900	304	0·47	35 957	25	463
8	10	26 500	265	0·69	38 406	22	493

Summary of procedures

Stage

1 Mucosa (1 l.) disintegrated in 1 l. distilled water and adjusted to pH 6·5.
2 *n*-Butanol (1·5 l.) added, vigorously stirred, and heated to 38° for 5 min., centrifuged at 1800 *g*, 40 min. Aqueous layer filtered through Hyflo Supercel.
3 Filtrate adjusted to pH 4·9 and filtered through Hyflo Supercel; filtrate adjusted to pH 8·6, held 12 hr. at 0°, refiltered as previously.
4 Enzyme (at pH 6·4) precipitated with ether (15 %, v/v) and acetone (52·5 %, v/v) at −5°. Precipitate dissolved in and dialysed against distilled water.
5 Solution at pH 4·9 heated to 48° for 2 min., cooled to −5°, adjusted to pH 6·4 and fractionated with ether (10 %, v/v) and acetone (35–40 %, v/v).
6 Dialysed enzyme solution fractionated at −5° with ether (10 %, v/v) and acetone (40–45 %, v/v). Precipitate dissolved and dialysed against distilled water.
7 Solution adjusted to pH 5·5 and treated with washed charcoal until about 30 % enzymic activity adsorbed.
8 Solution adjusted to pH 6·4 and fractionated with acetone (40–53 %, v/v) at −5°. Precipitate dissolved and dialysed against distilled water.

Table 6. *Summary of partial purification of alkaline phosphatases from ox kidney, liver and mammary gland*

	Procedure	Specific activities of fractions (units/mg. non-dialysable N) Kidney	Liver	Mammary gland
1	Aqueous suspension of washed particles	129	54	Not determined
2	Clear aqueous solution following *n*-butanol treatment	925	260	312
3	Solution heated, 42°, 5 min. at pH 6·8. Held pH 8·6, 8 hr., 2°	Not applied	Not applied	633
4	Precipitate of total phosphatase with ether and acetone, −7°	1300	Not determined	1780
5	Treatment at pH 4·9, 30 min., 18°, followed by pH 8·5, 12 hr., 0°	2800	930	Not applied
6	Fractionation with acetone at −5°	6900	2800	4400

directly to the 'homogenate' of intestinal mucosa in distilled water. Further fractionation gave preparations which were more active than those previously obtained by other workers. However, it was not possible to isolate the purified enzyme by this procedure, the maximum activity obtained in this way being 38 400 units/mg. N. This seems to be due to the failure to remove the soluble protein before release of the particle-bound enzyme. Apparently the soluble material contains a number of proteins which behave similarly to alkaline phosphatase during fractionation. Preparations by this method are nevertheless suitable for very many purposes and the enzyme is recovered in high yield (about 22 %). Moreover, the preparative procedures do not necessitate the use of large-capacity, or high-speed centrifuges. Therefore, an outline of the procedures used and the results obtained is given in Table 5.

Partial purification of the alkaline phosphatases of ox kidney, liver and mammary gland

Using procedures essentially similar to those described above, and on the basis of experience gained in purification of milk phosphatase (Morton, 1953*d*), alkaline phosphatases were partially purified from kidney, liver and mammary gland. In each case the phosphatase was obtained in solution by treatment of the aqueous suspension of precipitated particles with *n*-butanol. Further purification involved: (1) precipitation with ether and acetone at −7°; (2) exposure to pH 4·9 at room temperature (about 18°) for 30 min., and subsequently to pH 8·5 for 12 hr. at 0°; (3) fractionation with acetone at pH 6·4 and at −5°, retaining the most active fractions.

In this way preparations of high activity were obtained, as follows: kidney, 6900 units/mg. N (Q_p 47 800); liver, 2800 units/mg. N (Q_p 19 400) and mammary gland, 4400 units/mg. N (Q_p 30 500). However, the yields were rather low, viz. between 2 and 5 % of the initial material. The results are based on a single experiment in each case. The phosphatases from these tissues, like the similar enzyme from milk (Morton, 1953*c*), were found to be somewhat more labile than intestinal phosphatase. A summary of the results is given in Table 6.

DISCUSSION

Association of alkaline phosphatase with microsomal particles

While detailed distribution studies have not been carried out, it has been shown in this investigation that the specific activity of the microsome fraction,

both from calf intestine (Table 1) and cow mammary gland (Table 2) is very much greater than that of the mitochondrial fraction as well as of the whole tissue dispersion. This indicates that the alkaline phosphatase of these tissues is concentrated in the microsomes, and supports the earlier findings of Kabat (1941) with mouse kidney, Chantrenne (1947) with mouse liver, and Hers, Berthet, Berthet & Duve (1951) with both kidney and intestine of rabbit and guinea pig.

It has repeatedly been found that all the alkaline phosphatase of calf intestinal mucosa is removed by slow filtration of a tissue dispersion through a thick bed of Hyflo Supercel. This is not due to adsorption of the protein since the phosphatase, when in true solution following butanol treatment, is no longer retained by Hyflo Supercel. Thus it is concluded that all the alkaline phosphatase of intestinal mucosa is bound to particles. It is noteworthy that the recent studies of Novikoff, Podber, Ryan & Noe (1953) indicate that alkaline phosphatase of rat liver occurs partly in true solution and hence, in this respect, differs from the enzyme of kidney, mammary gland, milk and intestine.

The occurrence of microsomes in cow's milk suggests that some of these particles, like mitochondria, occur freely in cells (Morton, 1953*d*). However, it is likely that most of the heterogeneous microsome fraction isolated from tissue dispersions is derived by breakdown of other cellular structures. The phosphatase-rich brush border, intracellular membranes, and the so-called 'Golgi apparatus' probably contribute to the microsomes from intestinal mucosa. These particles, in contrast to microsomes from rat liver (Strittmatter & Ball, 1952), lack any red haemoproteins.

The effect of butanol on microsomal particles

During studies of the alkaline phosphatase in milk, it was found that this enzyme was firmly bound to lipoprotein particles ('milk microsomes') from which it could be quantitatively released into true solution by treatment with butanol (Morton, 1953*b*). The alkaline phosphatases of animal tissues may similarly be quantitatively released by treatment with butanol (Table 3). It is possible to use this treatment for assay of alkaline phosphatase in tissues, and the procedure has several advantages over use of whole tissue dispersions or autolytic extracts. The disadvantages of assaying alkaline phosphatase in tissue suspensions has been frequently noted (see Kay, 1932; Mathies, 1951). Moreover, as found by Mathies (1951) in the case of hog kidney, autolysis may not always liberate the enzyme from the particulate components. Further advantages of the use of butanol extracts for enzyme assay are: (1) Clear extracts are obtained, which retain their activity for long periods, and therefore may be stored for assay at a convenient time. (2) Natural inhibitors, if present, may usually be removed by dialysis. (3) Manipulations are simplified, and truly representative samples may be accurately prepared. (4) The butanol extract may be used for estimation of the total lipid and phospholipid of the material, if this information is required. Nucleic acid phosphorus and protein phosphorus may then be readily estimated without interference from lipid phosphorus.

The release of the alkaline phosphatase appears to be dependent on the disruption of lipid–protein bonds. The resistance of this enzyme to tryptic digestion (Schmidt & Thannhauser, 1943; Abul-Fadl & King, 1949*b*) permits the use of proteolytic methods for this purpose. However, it is most undesirable to use such procedures for purifying proteins, since (1) further protein (or proteins) are added, which must later be removed; (2) protein degradation products are formed, ranging from large polypeptides to peptides and amino acids, thus considerably increasing fractionation difficulties; (3) the enzyme to be purified may react with these products in a specific manner; and (4) the enzyme itself may be modified by proteolysis.

In contrast to butanol, other organic solvents such as chloroform, toluene, ether and acetone do not release alkaline phosphatase into true solution from aqueous suspensions of microsomal particles. The excess butanol recovered after treatment of microsomes as previously described has been found to contain predominantly phospholipids, mostly of lecithin and kephalin types with some acetal phospholipid, as well as a non-phosphorylated compound which induces ketonuria in rats (Stewart & Young, 1952). Much of the butanol-soluble material is precipitated, partly in crystalline form, when the butanol extract is held at $-15°$ for 24 hr.

Purification of alkaline phosphatases

Although the purification procedure has been described in detail only for the alkaline phosphatase of intestinal mucosa, experience with liver, kidney and mammary gland has shown that it may be used for purification of alkaline phosphatases of other animal tissues without major modification. The essential features of the purification method are: (1) Separation of the enzymically active particulate component as far as possible from soluble proteins and other material. (2) Release of the bound enzyme by treatment with butanol. (3) Fractionation with acetone (or acetone plus ether). (4) Selective denaturation of inert protein by treatment at pH 4·9 followed by treatment at pH 8·5. In the case of intestinal phosphatase, treatment at low pH should be combined with selective heat denaturation, viz. 48°, 2 min. (Table 4, stage 6). (5) Adsorption of inert protein on animal charcoal. In order to

obtain homogeneous preparations from tissues other than intestinal mucosa, it would be necessary to determine the most profitable sequence of these procedures.

The value of the early removal of soluble protein, followed by release of the enzyme by butanol treatment, is indicated by the sixty-fold purification gained at stage 3 (Table 4). However, the particles were obtained by repeated precipitation at pH 5·0. Undoubtedly the precipitate would contain protein, insoluble at this pH but soluble at pH 7·5, and mitochondria as well as microsomes. It is not surprising, therefore, that it was found in a small-scale trial that butanol treatment of washed microsomes, prepared by differential centrifuging at pH 7·2, gave a clear extract containing the alkaline phosphatase purified about 300-fold as compared with the whole 'homogenate'. The advent of centrifuges capable of handling relatively large volumes at high centrifugal forces has now made it possible to use such a procedure in preparative work, whereas this was not possible in 1949–50, when the work described here was completed. It is therefore strongly recommended that washed microsomes should be prepared by differential sedimentation as previously suggested (stage 2*a*), rather than by precipitation at pH 5·0. The remaining purification should then be carried forward as described.

During development of the purification method, a number of procedures were examined at each stage. In addition to fractionation with acetone and ether, low-temperature fractionation was examined using ethanol, *n*- and *iso*-propanol and dioxan, with and without addition of ether. None were as satisfactory as acetone, and some, such as dioxan, were found to be very toxic to the enzyme. Other adsorbents, such as calcium phosphate and alumina ($C\gamma$) gels, were found to be less effective than charcoal in removing inert protein. Adsorption of inert protein was found of much less value if used prior to stage 7 (Table 4). The enzyme could be readily adsorbed on calcium phosphate and alumina ($C\gamma$) gels, as well as on charcoal, and eluted with dilute salt solutions. However, if the enzyme were so treated after stage 7 (Table 4) the specific activity always declined. This suggests that there is some denaturation of the purified protein by spreading on the surface of the adsorbent. Hence adsorption and elution, such as used by Roche & Bouchilloux (1950), has been avoided in this purification procedure.

The concentrations of acetone given in the purification procedure (Table 4) should be used as a guide rather than as invariable limits, and it is recommended that small-scale trials should be carried out before treatment of the main bulk, in order to determine the most profitable fractionation limits. This also applies to stage 7 (Table 4), since the amount of charcoal to use is somewhat dependent on the impurities present after stage 6. Should the enzyme be adsorbed by using excess charcoal, it may be largely recovered by eluting 3 times with 0·25 M sodium carbonate containing 0·5 M ammonium sulphate and adjusted to pH 8·0.

Schmidt & Thannhauser (1943) obtained from calf intestinal mucosa a final preparation of specific activity 26 000 units/mg. N, and Roche & Bouchilloux (1950) modified the procedure of Thoai, Roche & Roger (1947) to obtain from dog intestine a preparation of specific activity 28 000 units/mg. N. From electrophoretic data, they considered this to be 51·7 % pure and so calculated that pure intestinal phosphatase should have a specific activity of 54 300 units/mg. N. It should be stressed that the high activities reported by Thoai *et al.* (1947) and Roche & Bouchilloux (1950) were obtained only after prolonged dialysis and reactivation of the enzyme with alanine. The best preparation obtained by Abul-Fadl, King, Roche & Thoai (1949), who also used dog intestinal mucosa but did not employ alanine re-activation, had a specific activity of 15 700 units/mg. N.

The preparation of specific activity 83 500 units/mg. N (Q_p 579 000), isolated from calf intestinal mucosa, is thus more active than any of the preparations described by other workers, and meets the criterion of purity, based on electrophoretic analyses, established by Roche & Bouchilloux (1950). Despite numerous attempts it has not been possible to obtain a more active enzyme. Homogeneity is also indicated by the absence of phosphodiesterase and pyrophosphatase activities, and of carbohydrate (Morton, 1952; cf. Schmidt & Thannhauser, 1943). Moreover, the nitrogen content (15·2 %) and the ultraviolet absorption spectrum (Morton, 1952) are also consistent with the preparation being a purified protein. Because of the limited amount of the purified material finally obtained, no attempt was made to crystallize the intestinal alkaline phosphatase. However, in view of the fact that microcrystalline material was obtained by Schmidt & Thannhauser (1943) with a preparation of considerably lower activity, it seems probable that conditions for crystallization could now be developed.

The preparations obtained from ox kidney, liver and mammary gland cannot be considered as homogeneous enzymes, although they are much more active than those hitherto obtained from these tissues. The enzyme from ox kidney, for example, has a specific activity of 6900 units/mg. N, and thus is about twice as active as the most active material previously prepared from the same source (Abul-Fadl *et al.* 1949). Similarly, the enzyme of specific activity 4400 units/mg. N obtained from mammary

gland was somewhat more active than the preparation of specific activity 3800 units/mg. N obtained by Caputto & Marsal (1941). However, further purification of the enzymes from kidney, liver and mammary gland was not pursued. An essentially homogeneous preparation of this class of alkaline phosphatases, which are distinct from intestinal alkaline phosphatases (Morton, 1952), had already been obtained from milk (Morton, 1953*c*). It has a specific activity of 15300 units/mg. N.

SUMMARY

1. The alkaline phosphatases of calf intestinal mucosa and of cow mammary gland are associated with microsomal particles.

2. By treatment with *n*-butanol at 35° the enzyme is readily obtained in true solution. On the basis of this effect a convenient method for assay of alkaline phosphatase of animal tissues is proposed.

3. The purification of alkaline phosphatase from calf intestinal mucosa is described. It involves butanol treatment of washed microsomes, followed by acetone precipitation, heating and charcoal adsorption.

4. The most active preparation had an activity (with β-glycerophosphate) of 83500 units/mg. N (Q_p 579000). This represents a purification of over 1000 times and thus is the most active phosphatase preparation as yet obtained.

5. The alkaline phosphatases of kidney, liver and mammary gland have been partially purified by similar methods.

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Copper Complexes in Grassland Herbage

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Copper deficiency disorders of cattle and sheep in Britain frequently exist in areas where the dietary copper intake of the grazing animal appears to be adequate. In areas where neonatal ataxia of lambs (Swayback) is found (a condition which can be prevented by copper therapy) herbage copper values are appreciably higher than those of areas in Australasia where the apparently identical syndrome, enzootic ataxia, is common. Thus Bennetts & Beck (1942) reported outbreaks of enzootic ataxia in Western Australia on pastures ranging in copper content from 1·1 to 6·1 p.p.m. (on dry matter) with a mean of 3·3 p.p.m. Cunningham (1944) described a copper deficiency in New Zealand where sheep

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have been grazing pastures with a copper content between 2 and 5 p.p.m. Beck (1941) has suggested that under Australian conditions pastures containing less than 5 p.p.m. copper are probably inadequate.

The following ranges have been quoted for the copper content of Swayback pastures in Britain, 14–27 p.p.m. (Innes & Shearer, 1940), 8–13 p.p.m. (Shearer, Innes & McDougall, 1940), 11–22 p.p.m. (Shearer & McDougall, 1944) and 6–13 p.p.m. (Mills, 1951). None of these workers have been able to show differences between the herbage copper values of affected and non-affected areas. Jamieson & Allcroft (1950), and Allcroft & Parker (1949) have described deficiency conditions in cattle which respond to copper therapy despite the fact that the copper levels in pasture ranged from 6·8 to 23 p.p.m.

From these results and from work which has been reviewed elsewhere (Allcroft, 1952; Marston, 1952) it is seen that many of the cases of copper deficiency in ruminants in Britain must be attributed to factors which either interfere with the copper metabolism of the animal or else affect the 'digestibility' of copper in the alimentary tract. The influence of molybdenum on the absorption and utilization of copper has been reviewed by Marston (1952). Dick & Bull (1945) have suggested that a high molybdenum intake may be a causative factor in conditioned copper deficiency disorders, but Stewart, Farmer & Mitchell (1946) have pointed out that pasture molybdenum values of affected areas of Britain are rarely above normal.

It was the object of the present work to investigate the possibility that the copper in herbage from affected areas may be present in a non-available form owing to the formation of stable organic complexes which the animal may be unable to absorb.

Many intermediates and products of plant and animal metabolism are structures with which copper can form complexes of considerable stability. Among such compounds are proteins (Mann & Keilin, 1938; Macheboeuf & Viscontini, 1943; Holmberg & Laurell, 1948; Klotz, 1952), amino acids (Albert, 1950*a*, 1952), porphyrins (Corwin, 1950), purines, pterins and flavins (Albert, 1950*b*, 1953; Mahler, 1953), catechol and its derivatives (Weinland & Walter, 1923) and tannins and lignin (Rademacher, 1936).

If copper is rendered unavailable to the ruminant by complex formation, the complexes produced must be sufficiently stable to resist breakdown by rumen micro-organisms and by the acid secretions of the abomasum. Furthermore, they must either be insoluble in water and fat or, if soluble, be of such large molecular size that passage through the wall of the digestive tract is prevented. That acid-stable complexes of copper exist in biological material has been demonstrated by Neish (1939) and Macheboeuf & Viscontini (1943). Schultze, Elvehjem & Hart (1934) have shown that a number of organic copper complexes are not utilized as sources of copper by the rat.

In the work to be described comparison has been made in all cases between herbage from normal areas and from areas where outbreaks of Swayback are common. Comparisons have been based on:

(1) The solubility of herbage copper in a series of organic solvents of increasing dielectric constant.

(2) The solubility of copper in water and in dilute solutions of tartaric acid and glycine. The object of using the last two solvents has been to enable a qualitative estimate to be made of the stability of the naturally occurring complexes by providing an excess of organic ligands with which the bound copper may be induced to chelate to form a soluble complex. Soluble copper has been separated from the herbage residue by dialysis.

(3) In a limited number of cases, water-soluble fractions of herbage have been examined by a chromatographic separation of ionic copper from copper present in the form of soluble complexes.

EXPERIMENTAL

Sampling and preparation of herbage. Samples were taken by hand plucking from fields on a Derbyshire farm where Swayback occurs regularly if copper administration to pregnant ewes is not carried out. Samples from a non-affected area were taken from a farm in Kent where this disorder was unknown. Herbage was rinsed in glass-distilled water as soon as possible after gathering to minimize soil contamination and then dried for 3·5 days at 60° in a current of air. The dried material was ground in a Christy & Norris Laboratory Hammer mill (screen 64) that had been carefully cleaned before use by milling filter paper. Ground samples were vacuum-packed in glass jars for storage. Glassware was rinsed twice with 70% (w/w) HNO_3, washed with glass-distilled water and tested for freedom from heavy metals by rinsing with a solution of diphenylthiocarbazone in CCl_4, prepared by the method of Parkes (1943).

Copper analyses. Cu was estimated colorimetrically as copper diethyldithiocarbamate using an adaptation of the method of Eden & Green (1940). Approximately 1 g. of dried plant material was taken for each analysis and digested in Pyrex glassware with 2 ml. 70% (w/w) HNO_3 (redist. A.R.), 1 ml. 98% (w/w) H_2SO_4 (A.R.) and 1 ml. 72% (w/w) $HClO_4$ (low Cu batches A.R.). After digestion 10 ml. 4% $Na_2P_4O_7$, $10H_2O$ (four times recryst.) were added followed by 5 ml. NH_3 solution (sp.gr. 0·880) and distilled water to bring to 25 ml. vol. 2 ml. 0·5% sol. of sodium diethyldithiocarbamate were added and the yellow copper complex was extracted into 2 ml. amyl alcohol (redist. A.R.) the alcohol layer being subsequently centrifuged at 1085 *g* for 5 min. to remove suspended water droplets. Colour measurement was carried out using a 'Spekker' absorptiometer (model H 760) with Ilford Spectrum Blue-Green filters no. 603 (max. transmission at 490–500 mμ.). Readings

were compared with a calibration curve obtained with standard solutions in a 1 cm. microcell. All analyses were duplicated and reagent blanks were performed with each batch.

Extraction of herbage with organic solvents. Seamless extraction thimbles were freed from Cu contamination by continuous washing for 24 hr. in 0·5 N-HCl (A.R.) and finally rinsed in glass-distilled water. Herbage (5 g.) was weighed into a dried thimble and placed in an all-glass Soxhlet apparatus. Serial extraction of each sample was carried out for 24 hr. periods with light petroleum (b.p. 40–60°), ether, $CHCl_3$, ethyl acetate, butanol and water in that order. The Cu extracted by each solvent and in the herbage residue was estimated as described. 'Water-soluble Cu' had to be determined by difference owing to losses due to frothing during concentration. All organic solvents were purified by distillation, fractions distilling outside a range of ±0·5° of the expected boiling point being rejected. Light petroleum and ether were dried initially with P_2O_5 and stored over sodium. $CHCl_3$ was dried and stored over P_2O_5 in a dark vessel. Ethyl acetate and butanol were stored over anhydrous $CaSO_4$.

Dialysis in aqueous solvents. Collodion dialysis thimbles were prepared by the method of Duckworth (1935). The membranes produced by this method were of satisfactory uniformity and proved to be equally permeable to cupric, cupriglycinate and cupritartrate ions. Membranes were stored under glass-distilled water containing a trace of toluene.

Duplicate samples of herbage were weighed into thimbles containing the following aqueous solvents. In the case of 'water-soluble Cu', 1 g. herbage was mixed with 20 ml. water in each thimble and dialysed for 36 hr. In the case of 'glycine-soluble Cu', 1 g. herbage was mixed with 20 ml. 0·02 M glycine and the thimble and contents were immersed in 80 ml. of this solvent for 24 hr. In the case of 'tartaric acid-soluble Cu' the procedure was the same as for the 'glycine-soluble Cu' except that the solvent consisted of 0·02 M tartaric acid. Dialysis was carried out with continuous stirring, against glass-distilled water in an all-glass apparatus through which a rate of flow of 5 ml./min. was maintained. In all cases the decrease in the content of Cu during dialysis was determined by analysis of the non-dialysable residue.

The chromatography of water-soluble components. The work of Dent (1949) and Crumpler & Dent (1949) in resolving α-amino acids as their copper complexes, and of Arden, Burstall, Davis, Lewis & Linstead (1948), Burstall, Davis & Wells (1949), Burstall, Davis & Linstead (1951) and Lederer (1948) on the filter-paper chromatography of inorganic ions suggested that a filter-paper strip technique might be developed for the chromatographic separation of free cupric ions and Cu organic complexes in aqueous herbage extracts. The suitability of a number of solvents for the separation of a range of Cu complexes and salts was examined. Butanol–5% acetic acid gave good resolution, but heavy 'tailing' of spots indicated that complex decomposition had been taking place during the run in the acid solvent. Benzene–water gave no evidence of decomposition but rather poor resolution. Phenol–water provided good resolution, and decomposition only occurred in runs with weak complexes. The results of separations achieved on a number of 18 hr. (descending) runs on Whatman no. 1 filter paper are given in Table 1.

Preparation of herbage extracts for chromatography. 2 g. samples of dried herbage were weighed directly into centrifuge tubes containing 50 ml. distilled water. Tubes were stoppered and shaken vigorously for 4 hr. on a 'Microid' flask shaker and then centrifuged for 18 hr. at 510 *g*. The clear supernatant was reduced to 1 ml. by drying in vacuum, first over $CaCl_2$ and finally over P_2O_5. 0·2 ml. of this concentrate was applied to the filter paper by means of an 'Agla' micrometer syringe on to forty marked spots. The solvent was allowed to descend for 18 hr. and the paper was then dried at 60°. Results were assessed by two methods, (i) by spraying with a reagent consisting of 4% $Na_2P_4O_7$, $10H_2O$, and 0·5% sodium diethyldithiocarbamate in NH_3 solution (sp.gr. 0·880); the position of the golden-yellow spots due to the Cu complex was marked and R_F values noted; (ii) by cutting the dried papers at right angles to the direction of solvent travel into five strips each of 0·2 unit of R_F; each strip was ashed in a muffle furnace and the ash extracted with 0·5 ml. 35% (w/w) HCl. The Cu content of this extract was determined by adding 1 ml. NH_3 solution (sp.gr. 0·880), 0·2 ml. 4% $Na_2P_4O_7$, $10H_2O$ and 0·2 ml. sodium diethyl dithiocarbamate, the Cu complex being extracted into 0·75 ml. amyl alcohol. Although method (ii) gave only a roughly quantitative assessment owing to the very small amounts of copper present, the results were of value in supplementing those obtained by visual examination of the chromatograms.

Table 1. *R_F values of copper complexes or salts in phenol/water as descending solvent on Whatman no. 1 paper* (18 *hr. at* 18°)

Organic ligand	R_F of copper complex or salt
Histidine	0·98
Tryptophan	0·98
Creatine	0·97
β-Phenylalanine	0·96
Proline	0·96
Methionine	0·95
Leucine	0·94
Alanine	0·90
Tyrosine	0·84
Glycine	0·60
Asparagine	0·59
Glutamic acid	0·39
Pyridoxin	0·94
o-Coumaric acid	0·93
Ethylenediamine	0·86
Catechol	0·83
Malic acid	0·63*
Pyruvic acid	0·62*
Ethylenediamine	0·35
Malonic acid	0·20*
Lactic acid	0·08*
Tartaric acid	0·08
Acetic acid	0·05
Citric acid	0·05
Oxalic acid	0·05
Succinic acid	0·05
Inorganic anion	**R_F of copper salt**
Nitrate	0·06
Sulphate	0·05
Chloride	0·05

* Indicates 'tailing' observed on all runs probably due to slow decomposition of complex during run.

RESULTS

Organic solvent extraction. The solubility of a compound in a solvent is largely related to the electrovalent or covalent character of the solute molecule and the dielectric constant of the solvent. As the electrovalent characteristics of a series of copper compounds decrease, their solubility in solvents of high dielectric constant also tends to decrease providing strongly hydrophilic groups are not introduced into the molecule. Thus if cupric ions in a biological system enter into organic-complex formation, the solubility of that copper in water may fall, while that in solvents of low dielectric constant may be significantly increased. Failing this, the complex will be insoluble in both aqueous and organic solvents.

The solvent distribution of dissolved herbage copper from 'normal' and 'affected' herbage (i.e. samples from Swayback areas) is given in Table 2. In all samples examined, a substantial proportion of the total copper remained insoluble in organic solvents and water. The insoluble residue from samples of normal herbage contained, on the average, 64 % of the total copper, the corresponding figure for affected samples being 67 % (calculated from Table 2). The mean values for copper soluble in light petroleum showed no significant differences between normal and affected samples (3·8 % as compared with 4·5 %). Ethyl acetate also showed no important differences (5·1 and 5·0 %). Extractions with ethyl ether dissolved about 0·6 % of the total copper in normal samples as compared with 2·3 % in affected samples. This difference is due to the slightly higher solubility of copper in samples gathered from affected pastures in early spring (samples *A* 1 to *A* 3). A similar seasonal variation was apparent in the figures for the chloroform-soluble copper of affected herbage at different stages of growth. Samples gathered in January showed a mean solubility of 3·5 % of total copper as compared with 0·5 % in June. No marked seasonal variations of the chloroform-soluble copper content of normal pasture was observed. The parallel variations in the figures for water and butanol extractions are not surprising since butanol with its high dielectric constant (17) would be expected to dissolve many water-soluble components of the herbage. Butanol and water together extracted more copper from normal samples (24 %) than from affected samples (17 %).

Considering all the samples together, approximately 13 % of the total copper in the herbage was dissolved by solvents of dielectric constant less than 10, a further 21 % of the total being extracted by butanol and water. This suggests that much of the readily soluble copper in herbage is combined with organic residues and is not in the form of inorganic salts.

Table 2. *The solubility of herbage copper in organic solvents*

Values for μg. soluble Cu/g. dry matter. Figures in parentheses express the percentage of the total Cu content.

Sample (no.)	Month of sampling	Total Cu	Light petroleum	Ether	Chloroform	Ethyl acetate	Butanol	Water (by difference)	Insoluble residue
'Normal' herbage									
N 1	February	6·50	0·25 (3·9)	0·08 (1·2)	0·04 (0·6)	0·46 (7·1)	0·85 (13·1)	0·99 (15·2)	3·83 (58·9)
N 2	March	7·25	0·27 (3·7)	0·05 (0·7)	0·08 (1·1)	0·36 (4·1)	0·90 (12·4)	1·05 (14·5)	4·54 (62·6)
N 3	March	8·75	0·30 (3·4)	0·04 (0·5)	0·09 (1·0)	0·22 (2·5)	1·04 (11·9)	1·00 (11·4)	6·06 (69·3)
N 4	May	7·30	0·44 (6·0)	0·03 (0·4)	0·20 (2·7)	0·35 (4·8)	0·70 (9·6)	0·52 (7·6)	5·06 (68·9)
N 5	June	5·75	0·11 (1·9)	0·03 (0·5)	0·16 (2·8)	0·39 (6·8)	0·98 (17·0)	0·58 (10·1)	3·50 (60·8)
'Affected' herbage									
A 1	January	8·10	0·26 (3·2)	0·14 (1·7)	0·20 (2·5)	0·37 (4·6)	1·24 (15·3)	0·28 (3·5)	5·61 (69·2)
A 2	January	6·25	0·28 (4·5)	0·27 (4·3)	0·38 (6·1)	0·27 (4·3)	0·64 (10·2)	0·46 (7·4)	3·95 (63·2)
A 3	January	5·50	0·33 (6·0)	0·24 (4·4)	0·36 (6·5)	0·21 (3·8)	0·55 (10·0)	0·37 (6·7)	3·44 (62·5)
A 5	June	7·30	0·44 (6·0)	0·04 (0·5)	0·06 (0·8)	0·32 (4·4)	0·80 (11·0)	0·20 (2·7)	5·44 (74·5)
A 6	June	7·10	0·21 (3·0)	0·04 (0·6)	0·05 (0·7)	0·56 (7·9)	0·76 (10·7)	0·56 (7·9)	4·92 (69·3)

Dialysis in aqueous solvents. Extraction of herbage samples with aqueous solutions was carried out by two methods:

(1) Extraction and dialysis with water alone. This procedure would be expected to remove any ionic copper which had escaped adsorption on insoluble cell materials and organic copper complexes that are readily soluble in water.

(2) Extraction with an excess of complex-forming agent followed by dialysis with water to provide qualitative information regarding the stability of copper complexes not extracted by procedure (1). This principle of competitive complex formation has been used by a number of workers to determine the order of affinity of a number of metal ions for an organic ligand (Pfeiffer, Thielert & Glaser, 1939; Ackermann, Prue & Schwarzenbach, 1949; Mellor & Maley, 1948) and of organic ligands for copper in an insoluble inorganic complex (Wain & Wilkinson, 1943).

If a complex-forming molecular species is introduced into a biological system and a water-soluble copper complex is formed which is then removed by dialysis, the extent to which copper is withdrawn from the system is governed largely by the relationship between the stability constants of the naturally occurring copper complexes and that of the complex formed with the added ligand (equation i).

$$\left[\frac{K_s \text{ Cu } B}{K_s \text{ Cu } A}\right]$$

$$\text{Cu } A + \text{complexing agent } B \rightleftarrows \text{Cu } B + \text{complexing agent } A \quad \text{(i)}$$

where A = naturally occurring ligands, and B = added ligand.

If 'bound' herbage copper remains insoluble in the presence of molecules with which it can form a stable complex it is an indication that the naturally occurring complexes are of great stability at that particular pH. Similarly, if the added ligand forms a weak complex and yet is capable of withdrawing copper from the system the preformed complexes must also be of low stability. In making comparisons of the performance of chelating agents in this way it is essential that the added solutions have the same effect on the pH of the system, otherwise the situation becomes complicated by changes in the degree of ionization of the organic ligands competing for the metal ion. Although this method does not enable an accurate assessment to be made of the stability of copper complexes in herbage it does

Table 3. *The solubility of herbage copper on extraction with water, glycine and tartaric acid*

Values for μg. soluble Cu/g. dry matter. Figures in parentheses express the percentage of the total Cu content.

'Normal' herbage Sample (no.)	Month of sampling	Total copper	Diffusible copper in: Water extraction	Glycine extraction	Tartaric acid extraction
*N*1	February	6·50	1·43 (22)	4·00 (62)	3·58 (55)
*N*2	March	7·25	1·38 (19)	2·05 (28)	1·74 (24)
*N*3	March	8·75	1·48 (17)	1·75 (20)	1·58 (18)
*N*4	May	7·30	1·10 (15)	4·24 (58)	2·20 (30)
*N*5	June	5·75	1·04 (18)	3·80 (66)	1·50 (26)
'Affected' herbage					
*A*1	January	8·10	0·49 (6)	2·50 (31)	2·11 (26)
*A*2	January	6·25	0·55 (9)	3·30 (53)	2·70 (43)
*A*3	January	5·50	0·49 (9)	2·66 (48)	2·36 (43)
*A*4	January	7·80	1·02 (13)	4·22 (54)	3·80 (49)
*A*5	June	7·30	0·60 (8)	1·97 (27)	1·38 (19)
*A*6	June	7·10	0·92 (13)	2·28 (32)	1·99 (28)

present a qualitative picture of the overall stability of these compounds.

The extractants used in these experiments were 0·02 M glycine (log K_s of Cu complex 15·4, Albert, 1950*a*) and 0·02 M tartaric acid (log K_s of Cu complex about 9–10, from results of Kahlenberg, 1895). Results are presented in Table 3. It will be seen that slight but consistent differences occur between the figures for water soluble copper in normal and affected samples. The average figure for normal samples is 1·29 μg./g. (or 18·2 % of the total), that for affected samples being 0·68 μg./g. (9·7 % of the total). No consistent variation in the water-soluble copper content was noted at different stages of growth. The average glycine-soluble copper content of normal samples in early spring (2·70 μg./g.) is approximately equal to that for affected samples at this stage of growth (3·22 μg./g.), but as the herbage matures differences become apparent as is shown in the comparison of results for samples *N* 4 and *N* 5 with *A* 5 and *A* 6, all of which were samples of late spring growth.

No appreciable differences appear to exist in the quantity of copper extracted by tartaric acid from normal and affected samples. Seasonal effects are slight, a small increase in the quantity of tartaric acid soluble copper being noted in the early spring samples *A* 1, *A* 2 and *A* 3.

The data in Table 3 suggest that, in the normal pastures examined, there is a pronounced variation in the stability of herbage complexes at different stages of growth. Examining this effect in detail it is seen that at a very early stage of growth (*N* 1) glycine and tartaric acid both extract an appreciable proportion of the total copper. As growth proceeds these extractants withdraw less copper, particularly in the case of sample *N* 3 (sampled March 1951 during a phase of rapid growth). In more mature herbage (*N* 4 and *N* 5) the solubility of herbage copper in glycine has increased much more than in tartaric acid. If these variations had been due to appreciable alterations in the free cupric ion concentration of cell constituents it would be expected that parallel changes would have been noted in the figures for water-soluble copper. As this was not the case it must be concluded that the changes are due to the formation of copper complexes within the herbage and that the stability of these complexes varies with the stage of growth of the plant. That variations of similar magnitude were not noted in affected samples is probably due to the fact that there was no period in the growth of this moorland herbage which corresponded to the 'lush' spring growth of the ley herbage from which normal samples were drawn.

Chromatography of water-soluble components. The results of an examination of the water-soluble fractions of two normal and two affected samples are presented in Fig. 1 and Table 4. The existence of a number of water-soluble copper complexes was demonstrated in each herbage examined. It appeared that at least eight compounds were present in the extracts of normal herbage as compared with five in the affected samples examined, the compounds giving spots of R_F 0·23, 0·37 and 0·77 being absent in the latter. From the data presented in Table 1 it is seen that inorganic copper did not give spots of R_F greater than 0·05, while of the twenty-eight water soluble complexes examined none had an R_F value of less than 0·08. Of the pasture samples examined *N* 2 and *N* 4 gave responses in the 0 to 0·05 range but such spots were almost entirely absent from samples *A* 5 and *A* 4. On the limited evidence available it would be premature to suggest that this indicated a low concentration of ionic copper in the extract of affected herbage although this may well be the case.

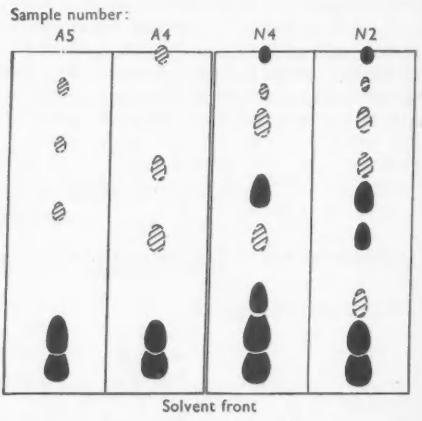

Fig. 1. Diagram of chromatograms obtained by running concentrate of aqueous extract of herbage with phenol–water (18 hr. at 18°). Sprayed with pyrophosphate–diethyldithiocarbamate–ammonia reagent. Intensity of shading indicates intensity of response.

Table 4. *Copper content of segments dissected from chromatograms* (μg. Cu × 10^2)

\+ Indicates that a positive response obtained but less than 0·03 μg. Cu present, and below the lower limit of detection using the Spekker absorptiometer.
\- Indicates no response for Cu obtained.

	R_F of segment analysed				
Sample	0·0–0·2	0·2–0·4	0·4–0·6	0·6–0·8	0·8–1·0
N 2	3	+	6	5	8
N 4	3	+	5	3	9
A 4	+	-	4	+	10
A 5	-	-	+	+	12

It is of interest to compare these results with those of Dawson & Nair (1950) who, using the copper-amalgam electrode have demonstrated the existence of two primary and four secondary complexes of copper in 'homogenates' of grass tissue.

DISCUSSION

The experimental data presented has shown that the greater part of the copper in herbage exists in a bound form and that much of this copper is not extractable by organic solvents or by dilute aqueous solutions of organic chelating agents. In no case have differences of great magnitude been demonstrated when comparing results for normal and 'swayback' herbage. The differences found have been small but reproducible and indicated that affected herbage contains less water-soluble copper probably both as cupric ions and as water-soluble complexes than normal herbage.

The extent to which the insoluble copper compounds of herbage would be affected by the hydrolytic and proteolytic activities of the digestive tract secretions and the action of rumen micro-organisms in the grazing animal is not known. If it is presumed that stable insoluble complexes are of low availability, the importance of small variations in the soluble copper content of the ingesta becomes more apparent. The problem therefore becomes reduced to these terms: is the lower content of soluble copper in affected herbage sufficient to induce a copper deficiency in the sheep? Before this question can be answered a number of points require investigation. Much more information is needed concerning the physiological availability of soluble copper complexes to the ruminant. Is cupric ion liberation from a complex a prerequisite to the absorption of copper from the digestive tract or can the intact molecule be utilized?

Comar, Davis & Singer (1948) and Mills (unpublished results), using ^{64}Cu, have demonstrated the very poor utilization of ionic copper administered orally to cattle and sheep. Havinga & Bykerk (1947) have shown that more copper is stored in the liver of calves after injection of stable soluble complexes of copper than after injection of equivalent quantities of copper as the sulphate.

In view of such observation it may well be that cupric ions are not as readily utilized as soluble complexes, for it is difficult to imagine that the free ion can be absorbed from the alkaline milieu of the small intestine rich in copper-binding groups and then pass through into the bloodstream without combining with immobile structural protein of the digestive tract wall. The work of Albert (1950*a*, *b*, 1951, 1952, 1953) has clearly demonstrated that in any biological system competition such as this must exist between metal ions and chelating groups of tissues and cell fluids.

Thus before full conclusions can be drawn from the results of this investigation the fundamental aspects of copper absorption and utilization by the animal require to be more fully understood. A study is being made of the dietary availability of copper complexes to the copper-deficient animal and the results of this work will be reported later.

SUMMARY

1. Herbage samples from both Swayback and normal pastures were subjected to organic solvent extraction with a series of solvents of gradually increasing dielectric constant. The quantities of copper extracted by different solvents are compared and the results discussed.

2. The solubility of herbage copper in water was investigated by the use of a dialysis procedure. The results indicate that rather less water-soluble copper is present in the Swayback pastures than in normal herbage.

3. Samples were extracted with dilute aqueous solutions of chelating agents. Seasonal variations in the solubility of herbage copper were demonstrated particularly in the case of the normal samples examined. The greater part of the copper in both Swayback and normal pastures appears to be bound in a form which is more stable than the copper-glycine complex.

4. The copper complexes present in the water soluble component of herbage were examined by the use of filter-paper partition chromatography.

5. The possible significance of the above findings is discussed in relation to the question of the availability of organic copper complexes to the ruminant.

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The Lipids of Fish

5. THE LIPIDS REMAINING IN THE FLESH OF THE HADDOCK AFTER EXTRACTION BY ACETONE AND ETHANOL–ETHER

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In the first paper of this series (Lovern, 1953) was described the preparation of a series of extracts from the flesh of the haddock, using solvents of presumed successively increasing effectiveness for lipids. The extracts obtained with acetone and ethanol–ether (3:1 by vol.), respectively, both at room temperature, have been discussed in earlier papers of this series (Lovern & Olley, 1953*a*, *b*; Olley & Lovern, 1953) and their extensive contamination with non-lipids was shown. The later extracts were obtained with ethanol–benzene (2:1 by vol.), chloroform–methanol (1:1 by vol.), both at the boiling point, and finally pyridine at 100°. Examination of these three extracts showed them to be overwhelmingly non-lipid in composition, and it was decided to present the results in a single paper. Moreover, in order to obtain sufficient lipid for detailed study, comparable extracts, available from an earlier series of experiments and since stored at −30° under nitrogen, were combined with the current series of ethanol–benzene and chloroform–methanol extracts.

ANALYTICAL METHODS

In general the analytical methods employed were as described in the previous papers. New features are given below.

Hydrolysis. The unsatisfactory hydrolysis of sphingomyelin with 0·5N ethanolic KOH (Lovern & Olley, 1953*b*) led to the use in the present work of 6N methanolic HCl for the hydrolysis (8 hr. refluxing) of fractions likely to contain over 5% of sphingosine. This reagent does not permit the separate isolation of unsaponifiable matter and plasmals, and the latter were assayed after such hydrolysis colorimetrically (Feulgen & Grünberg, 1938) on the unsaponifiable matter, separated from the methyl esters by a simplification of the procedure of Klenk & Friedrichs (1952). In all cases, however, only traces of plasmal were found, in contrast to the appreciable yields of supposed plasmal regularly isolated as a separate fraction after alkaline hydrolysis. Methanolic acid hydrolysis of ox-brain phospholipids readily gave the plasmal fraction in the form of dimethylacetals in the unsaponifiable fraction. This finding strengthens the doubts expressed in the last paper (Lovern & Olley, 1953*b*) whether the material isolated gravimetrically was genuinely plasmal. Its real nature remains obscure.

It has been found that unsaponifiable matter isolated after 2 hr. refluxing with 0·5 N ethanolic KOH is not in all cases resistant to more prolonged, e.g. 16 hr., refluxing with the same reagent. Thus, that portion of the pyridine extract which was insoluble in boiling chloroform–methanol (see below) yielded 161 and 98 mg. of unsaponifiable matter and fatty acids, respectively, after the customary 2 hr. hydrolysis period. A further 16 hr. hydrolysis of the former material gave 113 mg. unsaponifiable matter and a further 38 mg. of fatty acids. This resistance to hydrolysis of what we have designated 'wax' in earlier papers was noticed too late in the present work for all the unsaponifiable fractions to be so examined, but it was taken into account in assessing the likelihood of wax esters occurring in certain fractions. Such esters would probably give approximately equal amounts of acid and unsaponifiable matter on complete hydrolysis.

There is considerable controversy as to the conditions affording complete hydrolysis of sphingomyelin (Thannhauser, Benotti & Reinstein, 1939; Erickson, Avrin, Teague & Williams, 1940; Carayon-Gentil & Corteggiani, 1942; Artom & Fishman, 1943; Hack, 1946; Brante, 1949; Rennkamp, 1949). We have confirmed Rennkamp's finding that hydrolysis with methanolic HCl does not yield any free choline, but does quantitatively split the fatty acid–sphingosine linkage. We have further confirmed the observation of Brante (1949) that refluxing with saturated aqueous baryta quantitatively liberates the choline of sphingomyelin and we also observed quantitative liberation of inorganic phosphate. By contrast, hydrolysis with 0·5 N ethanolic KOH only gave low and variable yields of free choline, even after 48 hr. refluxing. Baryta hydrolysis (8 hr. refluxing) after a preliminary hydrolysis (8 hr. refluxing) with methanolic HCl did not give quantitative choline recoveries (yield of the order of 16% of theoretical), presumably because of the stability of the phosphorylcholine liberated by the acid hydrolysis (Rennkamp, 1949). Our final procedure for the hydrolysis of fractions likely to contain sphingomyelin therefore involved 8 hr. refluxing of one sample with saturated aqueous baryta, for choline determination and 8 hr. refluxing with 6 N methanolic HCl of another sample for determination of fatty acids and unsaponifiable matter. In contrast to ethanolic alkali and aqueous HCl (Lovern & Olley, 1953*a*; Olley & Lovern, 1953), methanolic HCl gave fatty acids (as methyl esters), entirely free from nitrogen.

The small quantities available for analysis sometimes required the determination of components in hydrolysates not primarily produced for that particular estimation. Thus serine, ethanolamine, inositol and glycerol were sometimes liberated by 8 hr. hydrolysis with 6 N methanolic HCl, removal of methyl esters of fatty acids and unsaponifiable matter with light petroleum, addition of 0·25 vol. water and a further 3 hr. refluxing. In the case of glycerol, it was necessary to allow for hydrolysis of glycerophosphate, which was found to proceed to the extent of 35% under these conditions. Glycerophosphate itself was always determined on the same (alkaline) hydrolysates as choline, but glycerol determination (Lovern & Olley, 1953*a*) required a minimum of 5 mg. glycerol, and hence could only be carried out on the main hydrolysis product, i.e. from 0·5 N ethanolic KOH or 6 N methanolic HCl according to sphingosine content.

Sphingosine. The method of McKibbin & Taylor (1949) is the only published procedure for the quantitative assay of sphingosine in lipids. As the authors point out, it depends on all non-sphingosine N becoming water-soluble during the hydrolysis. We have routinely assayed our fractions for sphingosine by McKibbin & Taylor's method, but there seems little doubt that in many cases the N found was not all sphingosine N (see below).

Reducing sugar. In the early part of the work reducing sugar was determined by the method of Brand & Sperry (1941) but this was superseded by the anthrone method of Mallov, McKibbin & Robb (1953), which proved far more satisfactory with our extracts.

EXPERIMENTAL PROCEDURE

The general procedure followed closely that described in the previous paper (Lovern & Olley, 1953*b*), in that the three extracts were freed from the greater part of the non-lipid contaminants by re-extraction with solvent followed by the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951) and (except in the case of the pyridine extract) later fractionated by countercurrent distribution between light petroleum (b.p. 40–60°) and 85% (v/v) aqueous ethanol. Additional fractionation steps included precipitation with acetone in the presence of $MgCl_2$ and separation of acidic lipids by washing an ethereal solution with aqueous Na_2CO_3. The examination of the three extracts will be described individually.

Ethanol–benzene extract

24 g. of older extract (*A*) and 79 g. of later extract (*B*) were available. Each of them was re-extracted with light petroleum (b.p. 40–60°) leaving 11·5 and 72·3 g. insoluble material, respectively. Both soluble and insoluble portions were taken up in chloroform–methanol (2:1 by vol.) and submitted twice to the procedure of Folch *et al.* (1951). The fractions insoluble in light petroleum each contained material insoluble in chloroform–methanol (approx. 2 and 10 g., respectively, for *A* and *B*), which proved to be entirely non-lipid in nature, since after alkaline hydrolysis it became completely water-soluble and yielded neither fatty acids nor unsaponifiable matter. Treatment according to Folch *et al.* yielded the following amounts of purified lipids: (*A*) 0·5 and 8·4 g.; (*B*) 3·1 and 6·4 g. from the petroleum-insoluble and petroleum-soluble fractions, respectively. These four fractions were combined to give 18·4 g. of lipid. The aqueous phases resulting from the four treatments were recovered and hydrolysed and the small yields (about 1% of each) of fatty acids and unsaponifiable matter obtained indicated that this material was mainly non-lipid. Extraction of the aqueous phase with light petroleum yielded a small amount (about 0·6%) of lipid which on hydrolysis yielded 65% of fatty acids and 41% of unsaponifiable matter.

The further treatment of the 18·4 g. of purified lipid is illustrated in Fig. 1*a*. Aqueous-alkali washing was confined to the acetone-soluble lipids, since any free fatty acids should occur there and, moreover, these fractions do not emulsify so readily as the acetone-insoluble ones. Pooling of fractions, on the basis of appearance, distribution curve, analytical data and spot tests (see previous papers) was as indicated in Fig. 1*a*. The countercurrent distribution curve is given in Fig. 2*a*. Cholesterol spot tests (fractions 20, 30 and 40) were all so faint as to be negligible; plasmal spot

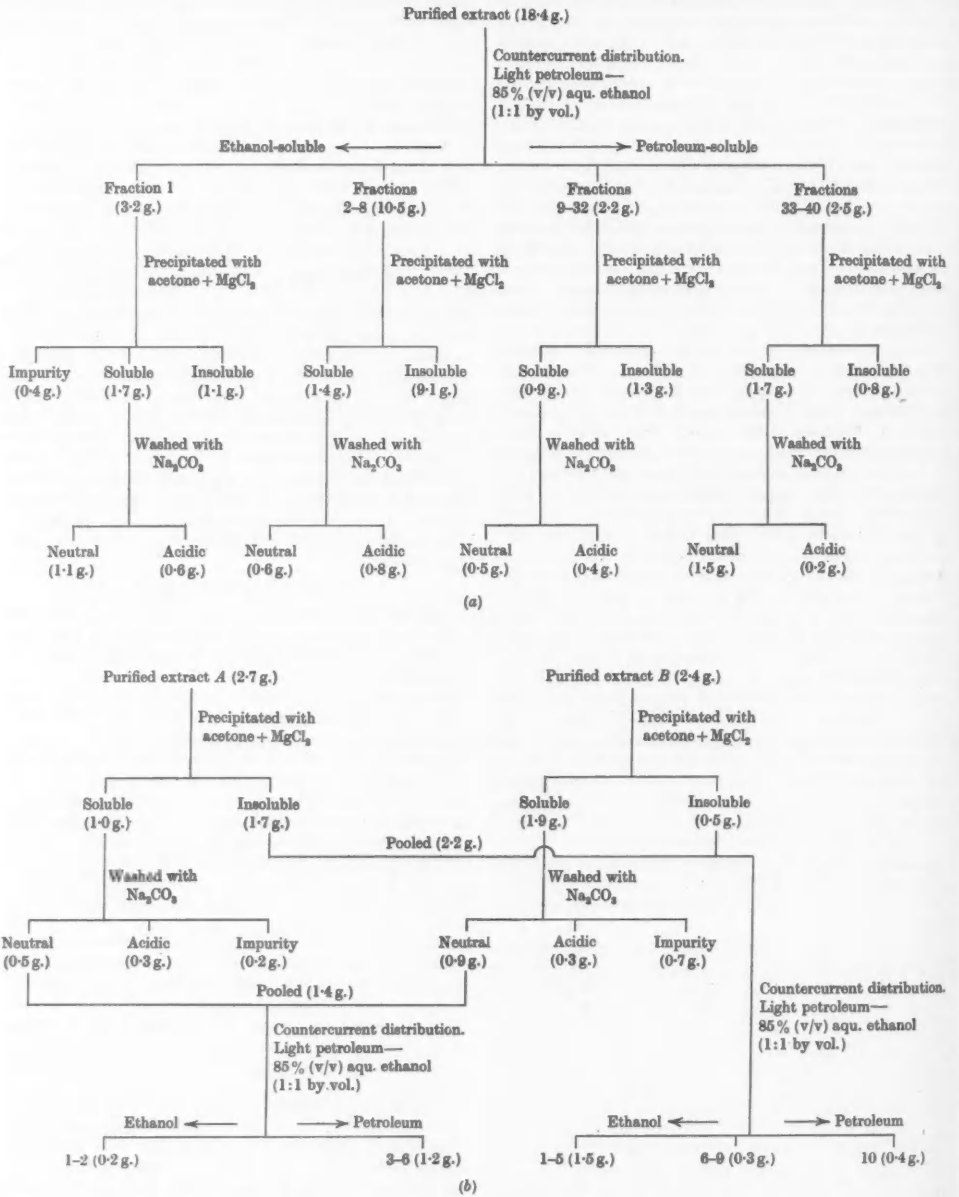

Fig. 1. Fractionation scheme for purified lipid extracts. All weights corrected for analytical and manipulative losses. (*a*) Ethanol–benzene extract; (*b*) chloroform–methanol extract.

tests (fractions 1, 10, 30 and 40) were all weak but quite definite. Fraction 1 proved to contain a small amount (0·4 g.) of material insoluble in organic solvents but soluble in water. It is designated 'impurity' in Fig. 1*a*, since on alkaline hydrolysis it yielded only 0·1% fatty acids and 0·2% unsaponifiable matter.

The acidic lipids did not resemble free fatty acids in appearance and proved to contain large amounts (1·3–2·6%) of phosphorus. They were, therefore, hydrolysed (0·5N ethanolic KOH) for analysis.

Chloroform–methanol extract

20 g. of older extract (*A*) and 103 g. of later extract (*B*) were separately re-extracted with chloroform–methanol (2:1 by vol.), leaving 6·45 and 44 g., respectively, of insoluble matter (1·45 and 0·2% unsaponifiable matter, 1·1 and 0·1% fatty acids, respectively). The chloroform–methanol solutions yielded (*A*) 2·7 g. and (*B*) 2·4 g. of purified lipids (chloroform phase) and the respective water-soluble impurities yielded 0·8 and 0·06% unsaponifiable matter and 0·6 and 0·07% fatty acids. The further treatment of these purified lipid fractions is shown in Fig. 1*b*. The material described as 'impurity' in the sodium carbonate washing process was a brown gum which separated when the acetone-soluble lipids were taken up in ether and which collected at the interface on washing with the aqueous Na_2CO_3. It yielded 11% unsaponifiable matter and 6% fatty acids on hydrolysis (0·5N ethanolic KOH), the rest of it becoming water-soluble. The acidic lipids were again rich in phosphorus (0·7–1·1%), not at all like free fatty acids in appearance, and were hydrolysed for detailed analysis. The countercurrent distribution curves for the neutral acetone-soluble lipids and the acetone-insoluble lipids are given in Fig. 2*b* and *c* respectively. The principle of pooling was the same as for the ethanol–benzene extract. Spot tests showed traces of cholesterol in pooled neutral lipids 3–6, but none in fractions 1 and 2. Traces of plasmal were detectable in both groups of neutral lipids and appreciable amounts in some of the acetone-insoluble lipids (fractions 6–10), but a negative plasmal reaction was obtained on fractions 1–5.

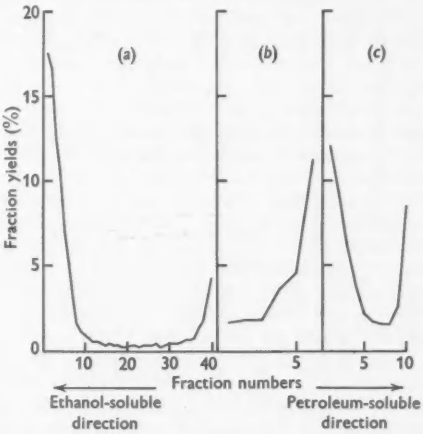

Fig. 2. Countercurrent distribution of lipid extracts of haddock flesh. Solvents 85% (v/v) aqueous ethanol and light petroleum (b.p. 40–60°). Ordinates: fraction yields (%). Abscissae: fraction numbers. Ethanol-soluble direction to left, petroleum-soluble to right. (*a*) Ethanol–benzene extract. Twenty flasks in series, 300 ml. light petroleum in each; lipid in flask 1. Twenty batches, each 300 ml., of ethanol passed from flask 1 to 20 and run off to give fractions 1–20. Petroleum remaining in flasks 20–1, in that order, gave fractions 21–40. (*b*) Acetone-soluble portion of chloroform–methanol extract. Six flasks in series, 100 ml. light petroleum in each; lipid in flask 1. Ethanol, in batches of 100 ml. passed from flasks 1 to 6, 1 to 5, etc., but no fractions run off. Flasks 6–1 gave fractions 1–6, in that order. (*c*) Acetone-insoluble portion of chloroform–methanol extract. Procedure as in (*b*) but with ten flasks in series, flask 10 yielding fraction 1.

Pyridine extract

65 g. of crude extract were re-extracted with boiling chloroform–methanol (2:1 by vol.), leaving 38 g. of insoluble material (0·3% unsaponifiable matter and 0·4% fatty acids on hydrolysis). The soluble material was treated twice by the procedure of Folch *et al.* (1951) giving 3·4 g. of purified lipids and 23·6 g. of water-soluble impurities, the latter yielding 1·5% unsaponifiable matter and 5·1% fatty acids on hydrolysis. The purified lipids were precipitated with acetone and $MgCl_2$ giving 3·3 g. of soluble and 0·1 g. of insoluble material. The former, on dissolving in ether and washing with aqueous Na_2CO_3 yielded 2·4 g. of neutral lipids, 0·4 g. of acidic lipids and 0·5 g. of a black tar which was insoluble in ether and water, but soluble in acetone. All fractions were hydrolysed for analysis, there being insufficient material for countercurrent distribution. Plasmal spot tests were negative on the neutral and acetone-insoluble lipids and a cholesterol test on the former was also negative. No spot tests were applied to the acidic lipids.

RESULTS

Analytical data on the various groups of fractions are given in Table 1. Blanks in the table indicate that the determination in question was not made, and question marks an unsatisfactory end-point. The nitrogen ascribed to ethanolamine contains minor proportions of serine nitrogen as shown by paper chromatography. Bound reducing sugar represents merely reducing sugar liberated by acid hydrolysis and there is no proof that it is a lipid component. In addition to the components listed in Table 1, which are all recognized constituents of one lipid class or another, most hydrolysates contained appreciable proportions of other types of fragment, e.g. acid-hydrolysable phosphate and volatile base (ammonia?). Fractions containing bound reducing sugar generally contained comparable proportions of free reducing sugar.

The data in Table 1 have been interpreted, so far as possible, on the lines adopted in previous papers, i.e. attempts were made to equate choline, ethanolamine, glycerophosphate and available fatty acids (in the light of claims for the latter by other com-

Table 1. *Analytical data on lipid fractions*

% Constituent in fraction. Blank values not determined.

Fraction	Fatty acids	Unsap.	Plasmals	Glycero-phosphate P	Choline	Ethanol-amine N	Sphingosine N	Inositol	Glycerol	Bound reducing sugar
Ethanol–benzene extract										
Acidic 1	49·0	15·8	3·0	1·64	2·3	0·03	—	Nil	—	—
Acidic 2–8	55·5	12·3	3·0	1·97	7·9	0·07	—	Nil	—	—
Acidic 9–32	47·0	26·4	1·0	0·98	0·3	0·02	—	Nil	—	—
Acidic 33–40	45·3	31·4	10·0	0·58	—	—	—	Nil	—	—
Neutral 1	47·0	←23·6→		2·94	Nil	0·02	0·32	Nil	—	0·07
Neutral 2–8	32·1	←21·0→		0·70	1·8	0·11	0·20	0·33	—	0·39
Neutral 9–32	24·0	69·0	10·0	Nil	—	—	—	—	1·26	Nil
Neutral 33–40	36·0	55·0	4·0	Nil	—	—	—	—	2·07	Nil
Acetone-insol. 1	12·9	←4·8→		2·95	6·3	0·16	0·28	1·9	—	0·42
Acetone-insol. 2–8	51·7	←2·9→		2·16	3·8	0·19	0·12	2·9	2·37	Nil
Acetone-insol. 9–32	20·8	11·0	4·8	1·50	1·7	0·11	0·13	0·33	0·88	Nil
Acetone-insol. 33–40	20·0	←12·2→		2·48	Nil	Nil	0·20	0·43	—	1·21
Chloroform–methanol extract										
Acidic *A*	53·2	25·6	5·9	0·78	0·7	0·03	—	Nil	—	—
Acidic *B*	45·2	37·5	5·9	0·53	0·7	0·03	—	Nil	—	—
Neutral 1–2	53·0	←41·0→		Nil	0·9	0·66	0·58	Nil	6·25	Nil
Neutral 3–6	14·4	←22·8→		0·56	0·3	0·43	0·21	Nil	0·56	Nil
Acetone-insol. 1–5	34·8	6·2	10·5	1·85	1·5	0·27	Nil	5·84	6·55	2·67
Acetone-insol. 6–9	48·5	←22·2→		0·77	0·2	0·12	0·17	1·97	1·40	1·42
Acetone-insol. 10	46·9	←32·0→		0·79	1·0	0·13	0·26	1·40	6·10	0·88
Pyridine extract										
Acidic	40·7	16·3	7·8	0·22	1·2	—	—	—	—	—
Neutral	16·1	←27·9→		Nil	2·7	Nil	1·03	Nil	—	2·0?
Acetone-insol.	13·1	←23·7→		3·6	2·1	0·23	0·26	1·54	—	?
Tar	17·0	25·0	—	0·10	Trace	—	—	—	—	—

pounds such as glycerol and unsaponifiable matter). In the previous paper (Lovern & Olley, 1953*b*) it was found more difficult to equate the various fragments for the ethanol–ether extract than had been the case for the acetone extract, and this trend is much more in evidence in the present work. Certain fractions clearly contained non-lipid forms of such compounds as choline and glycerophosphate, e.g. acetone-insoluble fraction 1 of the ethanol–benzene extract, where there is a gross deficiency of fatty acids. Similarly, neutral lipid 1–2 of the chloroform–methanol extract contains glycerol equivalent to about 60 % of triglycerides. (There is no inositol, so it cannot be assigned to inositol lipids.) Any triglycerides present should go to the extreme light-petroleum end of the countercurrent distribution, i.e. fraction 6. It has been found, however, that monoglycerides (α-monostearin) are concentrated at the ethanol end of the sequence, diglycerides (αα′-distearin)* behaving like triglycerides. The evidence seems insufficient at this stage to conclude that monoglycerides are present in these extracts. In all of such intractable cases, a choice has had to be made in assigning the available fatty acids among the possible lipids present, and where possible this has been influenced by information on countercurrent distribution behaviour and certain analytical values additional to those in Table 1. Thus in some cases, but not in all, there was sufficient unsaponifiable matter available for acetylation and determination of saponification equivalent, permitting an estimate of the relative amounts of alcoholic and non-alcoholic (hydrocarbon?) material. In other cases the unsaponifiable matter was assumed to be entirely alcoholic and, in general, alcoholic material was assumed to have been present as wax esters. An exception will illustrate how an 'informed guess' was sometimes made. Neutral lipid 1 of the ethanol–benzene extract contains glycerophosphate equivalent to about 45 % of fatty acids (found, 47 %), on the lecithin model, although in the absence of choline, ethanolamine and inositol, no known glycerophosphatide is present. This glycerophosphate must, however, be combined in some form of neutral lipid. If the unsaponifiable matter is assumed to require an equal amount of fatty acid to give wax esters, only some 23 % of fatty acids is available to go with the glycerophosphate. In previous experience (see, for instance, the subfractions of *S*1–5 in Olley & Lovern, 1953) we have noted a tendency for alcoholic unsaponifiable matter at the extreme ethanol end of the countercurrent sequence to be unaccompanied by equivalent amounts of available fatty acids, and it is probably in the free state. This would also be expected from structure and solubility considerations. Hence, in calculating the composition of the neutral lipid 1 in question, we have preferred, in contrast to the general procedure, to assign all the fatty acid to an unidentified lipid of a glycerophosphatidic nature, and to consider the unsaponifiable material as originally unesterified.

Fatty acids in excess of the requirements of other identified lipid fragments in any fraction have, as in previous papers, been assigned to 'unidentified lipid', using an arbitrary conversion value of 75 % fatty acids in the lipid. In some cases this might have contained glycerophosphate, in others it could not have done. It represents a general pool of unidentified, fatty acid-containing material and cannot be directly equated with any of the unidentified lipids *A*, *B* and *C* of the previous paper (Lovern & Olley, 1953*b*).

No direct use has been made of the values for sphingosine and bound reducing sugar. The highest sphingosine values were found in two acetone-soluble products (Table 1), whereas sphingolipids are, in general, considered to be insoluble in cold acetone. On the other hand, ceramides (Thannhauser & Fränkel, 1931; Fränkel & Bielschowsky, 1932), if present, would probably pass into the acetone-soluble material and Brante (1949) unexpectedly found this to be so for brain cerebrosides. But sphingomyelin and, probably, ceramides are concentrated toward the ethanol end of the countercurrent distribution sequence (cf. Lovern & Olley, 1953*b*; we have been unable to dissolve ox-brain cerebrosides in an 85 % ethanol–light petroleum solvent pair), whereas the sphingosine values in Table 1 show no marked trend in this direction. If the bound reducing sugar is a lipid component, it should show a parallelism to the sphingosine values, so far as is known from the chemistry of animal lipids (this argument would not apply to vegetable lipids). In fact there is no such parallel trend and the highest sugar content of all was found in a fraction (chloroform–methanol acetone-insol. 1–5) with no sphingosine.

The calculated compositions of the various groups of fractions and of the three total extracts are given in Table 2.

DISCUSSION

Non-lipids

The most striking result is the finding that these three extracts are predominantly non-lipid in nature. The subsequent insolubility of some of this material in chloroform–methanol is probably due to the smaller volumes of solvent used for re-extraction than for tissue extraction, but it should be noted that with the ethanol–benzene extract some of the non-lipid was soluble even in light petroleum, and

* Samples of mono- and distearin were kindly supplied by Dr M. L. Meara, of the Department of Organic Chemistry, Liverpool University.

Table 2. *Composition of fractions and total extracts*

As weights (g.) except final percentage for each extract.

Fraction	Lecithin	Phosphatidyl-ethanolamine	Inositol lipid	Plasmalogen	Unidentified lipid	Waxes and alcohols	Hydrocarbons, etc.	Triglycerides	Non-lipids
Ethanol–benzene extract									
Acidic 1	0·20	0·01	—	0·04	0·07	0·19	—	—	0·09
Acidic 2–8	0·32	0·02	—	0·05	0·14	0·20	—	—	0·07
Acidic 9–32	—	—	—	0·01	0·12	0·21	—	—	0·06
Acidic 33–40	—	—	—	0·04	0·03	0·13	—	—	—
Neutral 1	—	—	—	—	0·69	0·26	—	—	0·15
Neutral 2–8	0·08	0·04	0·01	—	0·04	0·14	0·05	—	0·24
Neutral 9–32	—	—	—	—	—	0·22	0·22	0·06	—
Neutral 33–40	—	—	—	—	—	0·63	0·51	0·31	0·05
Acetone-insol. 1	0·09	0·02	0·02	—	—	0·10	—	—	0·87
Acetone-insol. 2–8	2·38	0·93	1·26	—	1·47	0·52	—	—	2·54
Acetone-insol. 9–32	0·07	0·04	0·02	0·12	0·04	0·29	—	—	0·72
Acetone-insol. 33–40	—	—	0·02	—	0·07	0·19	—	—	0·52
Water-sol. 1	—	—	—	—	—	—	—	—	0·40
Total purified lipid	3·14	1·06	1·33	0·26	2·67	3·08	0·78	0·37	5·71
Total impurities removed	—	—	—	—	—	1·92	—	—	82·78
Total extract (103·1 g.)	3·14	1·06	1·33	0·26	2·67	5·00	0·78	0·37	88·49
Percentage composition	3·0	1·0	1·3	0·2	2·6	4·9	0·8	0·4	85·8
Chloroform–methanol extract									
Acidic *A*	0·03	0·01	—	0·04	0·06	0·16	—	—	—
Acidic *B*	0·01	0·01	—	0·03	0·02	0·22	—	—	0·01
Neutral 1–2	—	—	—	—	0·03	0·17	—	—	—
Neutral 3–6	0·01	0·13	—	—	—	0·13	0·21	—	0·72
Acetone-insol. 1–5	0·02	0·03	0·41	0·30	—	0·19	—	—	0·55
Acetone-insol. 6–9	—	0·02	0·03	—	0·06	0·13	—	—	0·06
Acetone-insol. 10	0·03	0·03	0·03	—	—	0·25	—	—	0·06
Total purified lipid	0·10	0·23	0·47	0·37	0·17	1·25	0·21	—	1·40
Total impurities removed	—	—	—	—	—	0·66	—	—	118·59
Total extract (123·45 g.)	0·10	0·23	0·47	0·37	0·17	1·91	0·21	—	119·99
Percentage composition	0·1	0·2	0·4	0·3	0·1	1·5	0·2	—	97·2
Pyridine extract									
Acidic	0·02	—	—	0·07	0·11	0·13	—	—	0·07
Neutral	—	—	—	—	0·35	0·24	0·55	—	1·26
Acetone-insol.	—	—	—	—	—	0·04	—	—	0·06
Tar	—	—	—	—	—	0·21	—	—	0·29
Total purified lipid	0·02	—	—	0·07	0·46	0·62	0·55	—	1·68
Total impurities removed	—	—	—	—	—	0·38	—	—	61·22
Total extract (65 g.)	0·02	—	—	0·07	0·46	1·00	0·55	—	62·90
Percentage composition	Trace	—	—	0·1	0·7	1·6	0·8	—	96·8

most of it in chloroform–methanol. As in the previous papers, non-lipid material was still present in the purified extracts and in nearly all of the final fractions. We have discussed previously (Lovern & Olley, 1953*b*) the possibility that some of this non-lipid may be present as a complex with one or other of the lipids. Recent reports, (Folch & LeBaron, 1953; Becker, Bode & Schrade, 1953) of the existence of phosphatide–peptide complexes in lipid extracts of various tissues may be noted. Our consistent finding (cf. Lovern & Olley, 1953*b*) of high proportions of (non-sterol) unsaponifiable matter in the small amount of lipid accompanying the non-lipids removed by the purification of Folch *et al.* (1951) suggests that this lipid (wax?) is an important component of lipid-peptide or similar complexes in fish muscle.

Acidic lipids

Another unexpected finding was the range and proportions of the products obtained on hydrolysis of the acidic lipids. These could certainly not have consisted of the usual type of phosphatidic acid. Interestingly, they were completely lacking in inositol and contained little more than traces of non-lipids, possibly owing to the additional possibilities of aqueous extraction. These acidic lipids gave perfectly clear solutions in aqueous sodium carbonate, from which they could only be extracted by ether after acidification. Exactly similar acidic lipids (which in this case were also tested for bound reducing sugar, present to the extent of 2–4 %) were obtained from comparable lipid extracts of cod flesh, in other studies now in progress in our laboratory. Again inositol was absent and non-lipids virtually so. It seems probable that complex formation occurs between a number of lipids in such a way that sufficient acidic groupings are free for the whole complex to form a water-soluble sodium salt. Appreciable proportions of both orthophosphate and unidentified phosphorus accompanied the glycerophosphate in the hydrolysates of acidic lipids.

Acetone precipitation

Although acetone precipitation in the presence of magnesium chloride, using optimum concentration of the latter (Sinclair & Dolan, 1942), does effect a marked concentration of the phosphatidic lipids, it by no means results in their quantitative recovery in the case of these particular fish lipids. It is possible that the unusually high degree of unsaturation of the fatty acids, noted in previous papers, is responsible.

Composition of haddock-flesh lipids

The very small amounts of lipid available in the various fractions prevented any attempt at sub-fractionation and there is no doubt that the assessment of their composition is, in many cases, less satisfactory than for the earlier extracts. Moreover, as already indicated, some of them evidently contained appreciable proportions of incomplete lipid structures, e.g. compounds lacking much of their fatty acid. All the lipids of the present series represent no more than traces in the flesh of the haddock. The older extracts (*A*) represented 20 kg. of fresh haddock tissue and the later ones (*B*) 42 kg. Hence the total tissue content of true lipid extracted, for instance, by ethanol–benzene was about 0·02 %, and any particular fraction or component represents a negligible proportion of the tissue. It is not, therefore, surprising to find such extracts containing major proportions of the trace constituents of muscle tissue, and there is no reason for regarding say, 'excess' choline or glycerophosphate as post-mortem artifacts.

Table 3. *Composition of the total lipids of haddock flesh*

	% total lipid
Lecithin	42·8
Unidentified lipid	15·2
Waxes and alcohols	10·5
Free cholesterol	6·1
Free fatty acids	6·1
Phosphatidylethanolamine	5·4
Inositol lipids	4·4
Cholesterol esters	3·4
Triglycerides	2·4
Hydrocarbons, etc.	2·0
Plasmalogens	1·7

Because of the negligible concentration of these three extracts in the original tissue, any errors in the assumptions made in this paper will not seriously affect an estimate of the total lipid composition of haddock flesh. A weighted summation of the compositions of the various extracts described in this and the previous papers is given in Table 3. In this summation allowance has been made for the subsequently-determined inositol content of the acetone extract (Lovern & Olley, 1953*b*), all unidentified lipids have been grouped together, and all non-lipids discarded. The outstanding importance of lecithins and, since the unidentified lipids are obviously a mixture, the role of waxes and alcohols as second largest constituent may be noted, while triglycerides are quantitatively among the least important components. The total lipid content of haddock flesh is now seen to be about 0·55 %.

Extractability of lipids

An indication of the degree of selectivity of the various solvents is given in Table 4. Whereas virtually all of some lipids, e.g. free and esterified cholesterol, and the greater part of others, e.g. lecithin, is extracted by acetone, this is not so for unidentified lipids, plasmalogens, waxes and hydrocarbons. Unsatisfactory though some of these

Table 4. *Proportion (%) of each lipid removed from tissue by successive solvents*

Lipid	Acetone	Ethanol–ether	Ethanol–benzene	Chloroform–methanol	Pyridine
Lecithin	88·5	9·8	1·6	0·07	0·03
Unidentified lipids	62·4	32·2	3·9	0·2	1·3
Waxes and alcohols	58·0	20·5	14·1	3·3	4·1
Free cholesterol	~100	—	—	—	—
Free fatty acids	~100?	—	—	—	—
Phosphatidylethanolamine	84·4	10·3	4·4	0·9	—
Inositol lipids	72·5	18·6	6·7	2·2	—
Cholesterol esters	~100	—	—	—	—
Triglycerides	96·5	—	3·5	—	—
Hydrocarbons, etc.	61·0	16·8	8·5	2·1	11·6
Plasmalogens	50·3	40·4	3·3	4·3	1·7

classifications may be in the present case, they do represent lipids with a different extraction pattern from the former types. In the case of the unidentified lipids, part of the effect is attributable to the 'unidentified lipid *C*' of the previous paper (Lovern & Olley, 1953*b*), which appeared as a major component exclusively in the ethanol–ether extract. A precisely similar appearance and distribution pattern of 'unidentified lipid *C*' has been observed in comparable extracts of cod flesh. The lipids most resistant to complete extraction are rich in non-sterol unsaponifiable matter, as reflected in the figures for waxes and hydrocarbons. Cioffi & Varetto (1952) have noted an increased proportion of unsaponifiable matter in the 'bound' portion of the lipids of *Penicillium javanicum*.

Many points remain unsettled from this series of studies, e.g. the precise nature of the various unidentified lipids and of the unsaponifiable matter. These will be the subject of future research.

SUMMARY

1. Haddock flesh, previously exhaustively extracted with acetone and ethanol–ether (3:1 by vol.) at room temperature, was extracted successively with boiling ethanol–benzene (2:1 by vol.), boiling chloroform–methanol (1:1 by vol.) and pyridine at 100°.

2. The three extracts proved to consist predominantly of non-lipid material, most of which was removed by partition between chloroform and water (Folch *et al.* 1951). The purified extracts were separated into acetone-soluble and acetone-insoluble fractions, and the former further subdivided into neutral and acidic lipids. Countercurrent distribution between light petroleum and 85 % (v/v) aqueous ethanol was also employed in fractionating the ethanol–benzene and chloroform–methanol extracts.

3. Acidic, neutral and acetone-insoluble fractions were all complicated mixtures, and the acidic lipids were neither free fatty acids nor simple phosphatidic acids. The amounts available precluded extensive subfractionation and estimation of composition from analytical data consequently suffered in precision.

4. It is, nevertheless, evident that the lipid portions of all these extracts differ appreciably from the previous extracts, in particular in the high content of unsaponifiable matter, for the present assigned to waxes and hydrocarbons.

5. The composition of the total lipids of haddock flesh, amounting, after purification, to 0·55 % of the fresh tissue, is assessed. Lecithins are outstandingly the major component (43 %) and triglycerides (2·4 %) quantitatively among the least important.

6. The solvents used showed a varying efficiency for the different lipids. Some, e.g. cholesterol, were almost entirely removed by acetone. Curiously, lecithin was removed by acetone to the extent of nearly 90 %. Some lipids were more resistant to acetone, and one component of the unidentified group was confined almost entirely to the ethanol–ether extract. The lipids most resistant to extraction are rich in non-sterol unsaponifiable matter, as are the traces of lipids associated with the water-soluble impurities removed during the purification processes.

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The Reaction of Haemoglobin and some of its Derivatives with *p*-Iodophenylhydroxylamine and *p*-Iodonitrosobenzene

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Evidence has recently been presented that *p*-iodophenylhydroxylamine ($I.C_6H_4.NHOH$) and *p*-iodonitrosobenzene ($I.C_6H_4.NO$) are produced during the metabolism of *p*-iodophenylurethane and *p*-iodoaniline in the rat (Crick & Jackson, 1952, 1953). These metabolites combine firmly with haemoglobin so that erythrocytes become effectively labelled when radioactive iodo-compounds are used. Concurrently, methaemoglobinaemia develops but disappears in the course of an hour or two without loss of radioactivity from the cells.

Phenylhydroxylamine ($C_6H_5.NHOH$) and nitrosobenzene ($C_6H_5.NO$) are known to induce methaemoglobin formation in red cells, and combination occurs between haemoglobin and nitrosobenzene (Heubner, Meier & Rhode, 1923; Jung, 1940). The reactions of haemoglobin and some of its derivatives with phenylhydroxylamine and nitrosobenzene have been studied spectroscopically by Keilin & Hartree (1943) who concluded that the complexes formed were unstable and easily dissociated.

Radio-isotope work described in this paper using $p[^{131}I]$-iodophenylhydroxylamine and $p[^{131}I]$-iodonitrosobenzene has shown that stable complexes are formed with oxyhaemoglobin, carboxyhaemoglobin and methaemoglobin, and that reaction may occur without apparent change in the absorption spectrum of the pigment. It seems likely that similar interpretations may apply to the reactions with phenylhydroxylamine and nitrosobenzene.

These reactions may be of importance in relation to the known toxic effects of aromatic nitro- and amino-compounds used in industry and medicine, for such substances may be metabolized to hydroxylamino- and nitroso-compounds which could be responsible for the observed toxic effects.

EXPERIMENTAL

The preparation of $p[^{131}I]$-iodophenylhydroxylamine (IPhNHOH) and $p[^{131}I]$-iodonitrosobenzene (IPhNO) has been described elsewhere (Crick & Jackson, 1953).

Crystalline rat oxyhaemoglobin. A modification of the method of Drabkin (1946) was used. The stroma from washed, haemolysed cells (1 vol. of cells to 10 vol. of water) was first removed by filtration through filter paper (Whatman, no. 30, double) and through a Seitz filter. Crystallization was induced by addition of solid $(NH_4)_2SO_4$ to opalescence, followed by refrigeration, and the product further purified by recrystallization three times from phosphate buffer (Sørensen, 0·067 M, pH 6·6).

Alkaline haematin. This was prepared by the method of Anson & Mirsky (1929–30) and twice recrystallized. For use it was dissolved in a mixture of acetone (1 vol.) and 0·5 M-K_2HPO_4 (3 vol.).

Reactions with IPhNHOH *and* IPhNO. Both IPhNHOH and IPhNO are virtually insoluble in water, and for use were dissolved in a minimal amount of absolute ethanol. The hydroxylamine is unstable in solution, especially in aqueous alcohol, and must be freshly prepared; the pure nitroso-compound is more stable. The technique used in studying their reactions with the pigments is illustrated by the following typical example:

A solution of oxyhaemoglobin in phosphate buffer (10 ml. of solution containing 580 mg. of pigment) was treated with a solution of IPhNHOH (25 mg. in 0·5 ml. of ethanol) and the mixture shaken for a few minutes. The violet solution was centrifuged to remove a small amount of debris and any excess reagent, and the supernatant treated with solid $(NH_4)_2SO_4$ added in small portions, to cause partial separation of the pigment. The latter was removed by centrifuging, dissolved in phosphate buffer (pH 6·6) and again partially precipitated. The separated pigment was again removed and dissolved in a suitable volume of the same buffer. From the radioactivity present in the solution (75 000 counts/ml.) and the specific activity of the IPhNHOH (104 counts/μg.), the amount of this latter substance associated with the pigment

was calculated (721 μg./ml.). Since the Fe content of the solution (89 μg./ml.) was known the combining ratio of the pigment and IPhNHOH (Fe:IPhNHOH) could be calculated (89/56:721/235 in the example, or 1:1·94). The process of solution in buffer and partial precipitation with $(NH_4)_2SO_4$ was repeated as necessary to a constant combining ratio.

Estimation of radioactivity was made by the liquid counting technique, whilst Fe was determined in samples ashed in a Pt basin using thiolacetic acid (Jackson, Klein & Wilkinson, 1935). A quartz spectrophotometer (Unicam) was used for spectrophotometric measurements; solutions for these were adjusted so that the concentration of iron was always 400 μg./100 ml.

RESULTS

The reaction of oxyhaemoglobin with p-*iodophenylhydroxylamine and* p-*iodonitrosobenzene*

Except where specifically stated, IPhNHOH and IPhNO labelled with radioactive iodine were used in the reactions described below. Addition of one molecular equivalent of either IPhNHOH or IPhNO to a solution of crystalline oxyhaemoglobin caused the rapid formation of spectroscopically similar violet-red pigments, I (Fig. 1), with combining ratio 1:1 (Table 1). This material did not react further with added IPhNO, but rapidly combined with IPhNHOH (1 mole equivalent) to form a more intensely violet pigment, II (Fig. 1 and Table 1). The absorption spectrum of this substance soon changed on standing, owing to the formation of a third compound, III (Fig. 1), without loss of combined radioactive material (Table 1). This substance underwent a final slow transformation to a brown pigment, IV (Fig. 1), spectroscopically similar to methaemoglobin, with the loss of one half of the associated radioactivity; the final combining ratio was thus 1:1 (Table 1). This material, IV, remained stable for some weeks at least without change in the combining ratio.

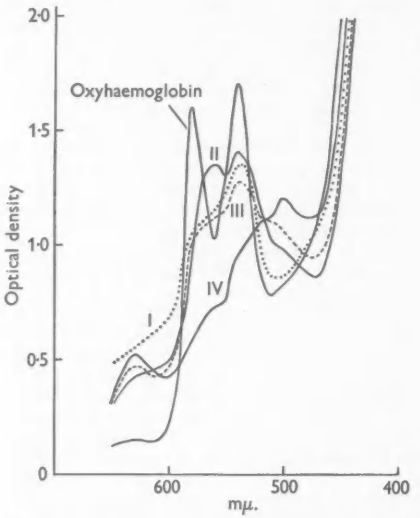


Fig. 1. The absorption spectra of four complexes formed consecutively after the addition of *p*-iodophenylhydroxylamine to a solution of crystalline rat oxyhaemoglobin.

Excess of IPhNHOH added to oxyhaemoglobin solution yielded compound II directly; excess of IPhNO gave I only. The latter substance slowly changed on standing into methaemoglobin-like pigment without loss of radioactivity, so this end product is presumably IV. Addition of an excess of IPhNHOH to substances III and IV caused their conversion back into II (Fig. 2), changes which must be due to the reducing power of the hydroxylamine.

Two experiments were set up as follows: (*a*) oxyhaemoglobin solution (1 mole equivalent) +IPhNHOH, radioactive (1 mole equivalent); (*b*) oxyhaemoglobin solution (1 mole equivalent) +IPhNHOH, inactive (1 mole equivalent). Compound I was formed in each instance, radioactive in (*a*) and inactive in (*b*). To the product from reaction (*a*) another molecular equivalent of inactive IPhNHOH was added, and a similar amount of radioactive IPhNHOH to (*b*). The reactions were allowed to proceed via the complex III to the final stage (compound IV) and the methaemoglobin-like pigment separated in each case. That from reaction (*a*) proved to be radioactive (combining ratio, 1:1·2), whilst (*b*) furnished pigment free from radioactivity. The relative amounts of radioactivity, associated with comparable solutions of the two pigments were 33 700 counts/ml. for (*a*) and 320 counts/ml. for (*b*). These two experiments clearly establish that the IPhNHOH combining in the reaction I → II (Fig. 2) was subsequently lost in the conversion of the pigment III → IV (Fig. 2); also that adsorption of radioactive material during the separation of the pigment complexes was not likely to be a complicating factor in these reactions in general.

Compounds I, II and III were easily reduced by dithionite to pigments spectroscopically similar to haemoglobin (V and VII, Fig. 2), which could then be oxygenated to red pigments (VI and VIII, Fig. 2) with the absorption maxima of oxyhaemoglobin (Fig. 3 and Table 1), all without change in the amount of associated radioactive material. The red, oxygenated pigment solutions slowly oxidized in air (as does oxyhaemoglobin itself) to the brown pigment IV (combining ratio, 1:1).

Since the oxygenated pigment, VIII (Fig. 2), was believed to contain only —NHOH groups, the following experiment was undertaken to find out whether the IPhNHOH molecules were attached to

spatially equivalent positions in the haemoglobin molecule. A solution of oxyhaemoglobin was treated with a slight excess of inactive IPhNO (1·1 mole equivalents), and to the violet product (I) a further molecular equivalent of radioactive IPhNHOH was added. The pigment (II) so formed was then reduced with dithionite, oxygenated, and allowed to stand until conversion into IV was complete. The brown pigment was separated in the usual manner and was found to be free from radioactivity. If the IPhNHOH molecules were attached in a symmetrical manner the loss of only one half of the radioactive molecules would be expected in the change VIII → IV (Fig. 2). As all the radioactive molecules were lost, it may be concluded that the attached IPhNHOH molecules in compound VIII were differently situated.

Reactions with carbon monoxide

The reactions of the various pigment complexes referred to above with carbon monoxide were also examined. Compounds I, II and III reacted directly with the gas, forming carboxyhaemoglobin-like pigments (X, XII and XIII, Fig. 4; see also Fig. 5) without change in the combining ratio, Fe/radioactive iodine (Table 1). The complex XIII, unlike the others, possessed an absorption maximum at 630 mμ. in addition to those characteristic of carboxyhaemoglobin. This absorption maximum was also present in the parent compound III. Both oxygenated pigments VI and VIII also combined readily with carbon monoxide, forming derivatives spectroscopically similar to carboxyhaemoglobin without loss of radioactive material (XI and IX,

Table 1. *Derivatives of haemoglobin combining with* p-*iodophenylhydroxylamine and* p-*iodonitrosobenzene*

For explanation of Table see text. No estimations were carried out on compounds V and VII, but their combining ratios may be inferred, since the ratios of the antecedent complexes (I and III) were the same as those of succeeding derivatives (VI and VIII respectively).

Compound	Suggested formula	Combining ratio (molecules I-compound/atom Fe)	Absorption maxima (mμ.)
I	Hb.IPhNO	0·86 1·1 1·0 1·1 1·2	— 538
II	Hb(IPhNO)(IPhNHOH)	2·0 1·8 1·8	560 538
III	Hb(IPhNO)(IPhNHOH) (ferric)	2·1 2·0 2·0 1·8	630 538
IV	MHb.IPhNO (ferric)	0·89 1·0 1·0 1·2	630 500
V	Hb.IPhNHOH	—	— 560
VI	HbO_2.IPhNHOH	1·0 1·2	580 540
VII	Hb.$(IPhNHOH)_2$	—	— 560
VIII	$HbO_2(IPhNHOH)_2$	1·8 1·9 1·8	577 539
IX	COHb.$(IPhNHOH)_2$	1·8 1·8 1·9	570 538
X	COHb.IPhNO	0·83 0·94	570 538
XI	COHb.IPhNHOH	1·1 1·2	570 538
XII	COHb(IPhNO)(IPhNHOH)	1·8 1·8	570 539
XIII	COHb(IPhNO)(IPhNHOH) (ferric)	1·8 —	630 570 540
	HbO_2	—	577 539
	COHb	—	570 538
	Haematin.IPhNO	1·1 0·87 1·3 0·83	

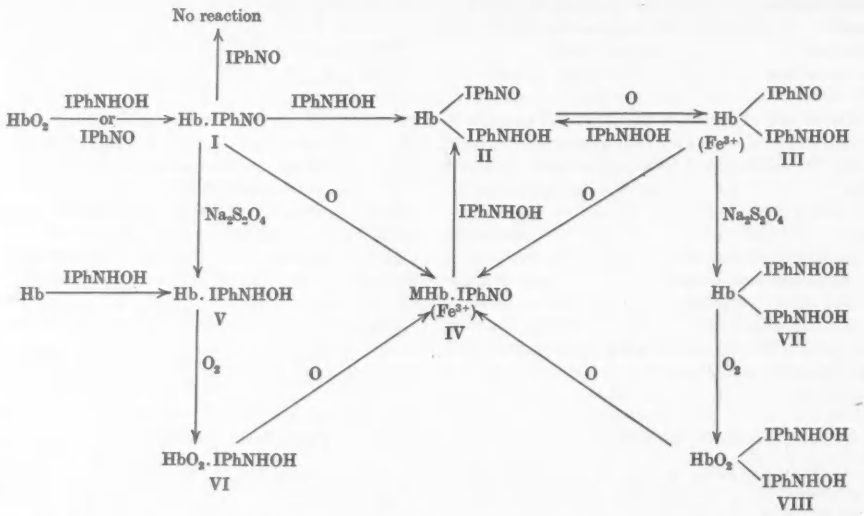


Fig. 2. Proposed scheme for the reactions of oxyhaemoglobin and haemoglobin with *p*-iodophenylhydroxylamine (IPhNHOH) and *p*-iodonitrosobenzene (IPhNO). Except in the complexes III and IV, the iron atom is considered to be Fe^{2+}.

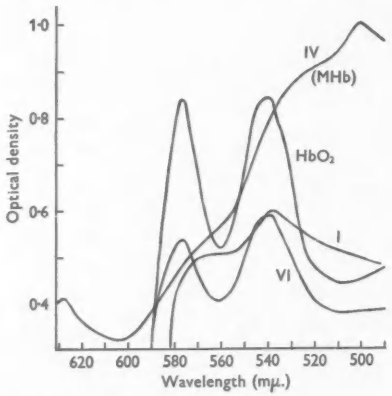


Fig. 3. The absorption spectrum of oxyhaemoglobin compared with HbO_2.IPhNHOH (VI) formed by reduction and oxygenation of Hb.IPhNO (I). Both complexes slowly oxidize to MHb.IPhNO (IV), the absorption curve for which is presented with a modified ordinate.

Fig. 4 and Table 1). The compounds IX and XIII separated in crystalline form, although no serious attempt was made to prepare the derivatives in this state.

Carboxyhaemoglobin itself reacted directly with IPhNHOH or IPhNO without change in absorption spectrum (IX and X, Fig. 4 and Table 1) so that no reaction would be suspected in the absence of a radioactive-tracer technique. Two molecular equivalents of IPhNHOH per atom of iron combined, but only one of IPhNO (Table 1). Quantitatively these reactions resemble those between oxyhaemoglobin and the two iodo-compounds, but oxidation of IPhNHOH to IPhNO could not occur in the reaction of the hydroxylamine with carboxyhaemoglobin, since an atmosphere of carbon monoxide was maintained throughout. IPhNHOH can therefore react directly with haemoglobin and prior or simultaneous oxidation to the nitroso-compound need not occur. Confirmation of this was sought in the following manner. A solution of oxyhaemoglobin was completely reduced with excess dithionite and IPhNHOH (1 mole equivalent) added. When the mixture was oxygenated the colour soon changed to bright red and spectroscopic examination of the solution gave the sharp absorption bands of oxyhaemoglobin. The separated pigment proved to be radioactive with a combining ratio of 1:1 (VI, Fig. 2). If reaction between the haemoglobin and IPhNHOH had not occurred before oxygenation, then the formation of the violet-red pigment I would be expected.

Reaction of haematin with p-*iodophenylhydroxylamine and* p-*iodonitrosobenzene*

Removal of the globin from the pigment complex III by means of acetone–hydrochloric acid mixture as specified by Anson & Mirsky (1929–30) was not

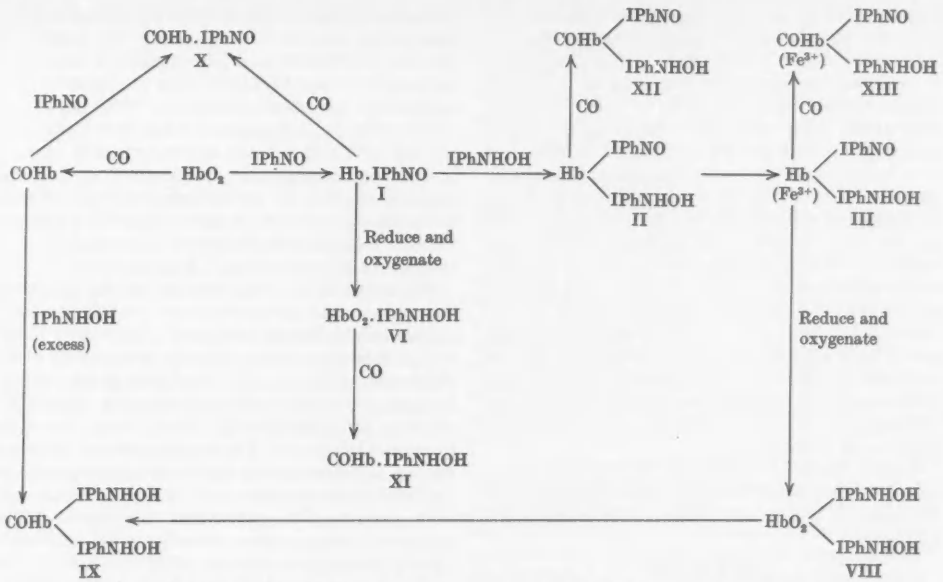


Fig. 4. Reactions of various pigment complexes referred to in Fig. 2 (I, II, III, VI and VIII) with carbon monoxide. The combination of carboxyhaemoglobin with IPhNHOH and IPhNO produces two complexes which fit into this scheme (IX and X).

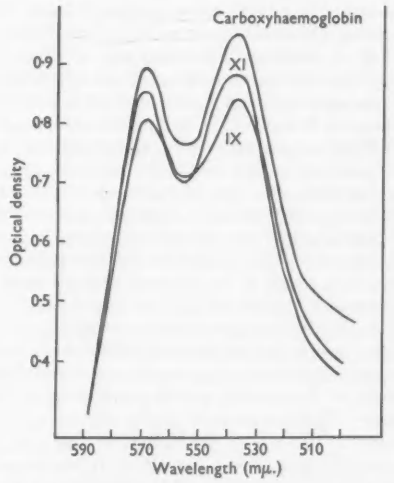


Fig. 5. Absorption spectrum of carboxyhaemoglobin compared with those of the complexes COHb.$(IPhNHOH)_2$ and COHb.IPhNHOH (IX and XI respectively). All the carboxy-pigments showed similar spectra (see Table 1), but the compound XIII showed an additional maximum at 630 mμ.

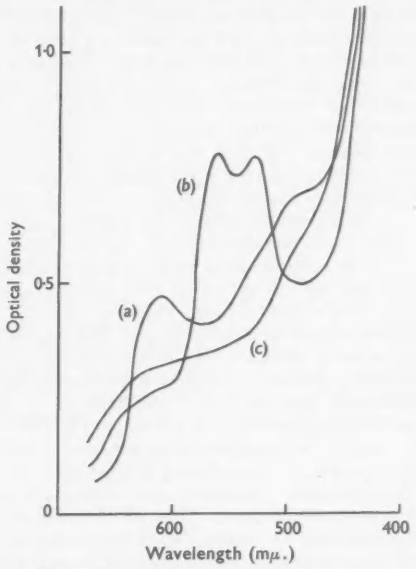


Fig. 6. Alkaline haematin (*a*) reacts with an excess of iodophenylhydroxylamine with the immediate formation of a bright violet-red pigment (*b*). This quickly changes on oxygenation to a greenish yellow material (*c*), with a combining ratio of Fe:IPhNHOH of 1:1. Iodonitrosobenzene added to alkaline haematin causes no visible colour change and the substance (*c*) is formed directly.

accompanied by any significant loss of radioactive material; 92% remained associated with the pigment in the protein-free solution. Alkaline haematin in acetone–phosphate buffer solution (pH 9·0) reacted rapidly with IPhNHOH and IPhNO. When IPhNHOH was added to the greenish yellow haematin solution the colour changed immediately to a bright red-violet with absorption maxima at 560 and 530 mμ. (Fig. 6). This compound was very unstable and the solution rapidly became greenish yellow again, especially on oxygenation. The final pigment was not haematin according to spectroscopic data and was found to retain one molecular equivalent of radioactive material (Table 1). No obvious change in colour occurred when IPhNO was added to a solution of alkaline haematin, but the separated product was similar spectroscopically and in its content of radioactive material to that from the reaction of alkaline haematin and IPhNHOH (Fig. 6 and Table 1).

Failure of either IPhNHOH or IPhNO to react with protoporphyrin 7-methyl ester suggested that the presence of iron was necessary for combination to occur.

DISCUSSION

The results presented show that oxyhaemoglobin, carboxyhaemoglobin and alkaline haematin react directly with *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene, and the questions arise as to the nature of the complexes and the mechanism by which they are formed. Keilin & Hartree (1943) studied the reactions of oxyhaemoglobin and alkaline haematin with phenylhydroxylamine and nitrosobenzene using a spectroscopic technique. They concluded that the violet pigment formed from PhNHOH and oxyhaemoglobin was very unstable, being decomposed in air into free methaemoglobin, oxidized by ferricyanide to methaemoglobin, reduced by hydrosulphite to haemoglobin and converted by carbon monoxide into carboxyhaemoglobin. The violet haematin complex produced by the reaction of alkaline haematin and PhNHOH underwent a similar series of changes, liberating haematin, haem, or forming carbon monoxide haem as the case may be. Some modification of their conclusions concerning the nature and stability of the complexes is suggested by the results of the present work with the labelled IPhNHOH.

An important conclusion which emerges is that the presence of combined IPhNHOH or IPhNO in the pigment molecule may be without effect on the absorption spectrum. Thus, spectroscopic data may neither indicate if reaction between the haemoglobin pigment and these aromatic molecules has occurred, nor show if the complexes formed have been dissociated when they are subjected to some simple chemical procedure (e.g. oxygenation, reduction, oxidation, etc.) which causes a change in absorption spectrum. According to Keilin & Hartree, the formation of violet pigment from oxyhaemoglobin and PhNHOH was preceded by the appearance of methaemoglobin. We have not observed the prior formation of this brown pigment when IPhNHOH was added to solutions of crystalline rat oxyhaemoglobin. It appears to us that the initial formation of methaemoglobin occurred in red cells or with less-purified pigment solutions, when it is apparently catalysed by the presence of the hydroxylamine (Crick & Jackson, 1953).

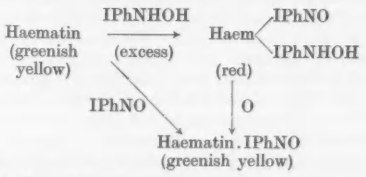
Whilst we have no information on the nature of the union with IPhNHOH or IPhNO in these pigment complexes, sufficient data have been accumulated to enable a scheme of reactions to be suggested (Figs. 2, 4). Oxyhaemoglobin reacts stoicheiometrically with one molecular equivalent of either IPhNHOH or IPhNO to form the same pigment complex (I). During the reaction with the former substance, the hydroxylamino group is oxidized to nitroso, although this does not mean—as was previously thought—that the haemoglobin molecule cannot react directly with the aryl hydroxylamine (see below). Reaction of IPhNO with oxyhaemoglobin is accompanied by evolution of all the bound oxygen; this has previously been found to occur when nitrosobenzene was used (Heubner *et al.* 1923; Jung, 1940). The fact that compound I does not react further with IPhNO but rapidly takes up an additional molecule of IPhNHOH suggests the formulation II, the iron atom remaining in the ferrous state. Both these complexes (I and II) are reduced by dithionite yielding a haemoglobin spectrum, so that this change can only represent a reduction of nitroso- to hydroxylamino-groups with the formation of compounds V and VII. The presence of combined IPhNHOH apparently does not interfere with subsequent oxygenation of these haemoglobins, for red pigments are then formed (VI and VIII) spectroscopically similar to oxyhaemoglobin (Fig. 3 and Table 1). The process of oxygenation does not cause oxidation of the unstable —NHOH groups to —NO, i.e. there is no reverse change back to compound I which would be expected if this happened. The complex II is unstable and soon changes into a third substance, III, without loss of the combined iodo-component, and this change appears to be due to the formation of a ferric pigment. This is supported by the appearance of an absorption band with maximum at 630 mμ., the observation that this pigment (III) is converted back into II by addition of IPhNHOH (obviously a reduction) and the knowledge that only one molecule of IPhNO can be attached to haemoglobin. The slow conversion of III on standing, into a methaemoglobin-like pigment could then be due

to the gradual oxidation of the —NHOH to —NO, leading to the loss of one of the two molecules of IPhNO. Preparation of compound III with one of the iodine components labelled, followed by its conversion into IV, demonstrated that the IPhNHOH molecule is specifically lost in this change. The substance VIII, when similarly labelled, also lost the corresponding molecule of IPhNHOH on oxidation to IV. These results suggest that the two molecules of combined iodo-compound are not symmetrically disposed in the haemoglobin molecule. The complex I is a ferrous pigment, for it slowly oxidizes to the methaemoglobin pigment IV without loss of combined IPhNO (Fig. 2). Addition of excess of IPhNHOH to IV converts it back into II, the hydroxylamine causing a reduction of ferric to ferrous in this reaction.

Pigments spectroscopically similar to carboxyhaemoglobin are formed by the action of carbon monoxide on the substances I, II and III without loss of combined radioactive material (Figs. 5, 4). The fact that IPhNO also reacts directly with carboxyhaemoglobin without causing a change in absorption spectrum, while it releases all the oxygen from oxyhaemoglobin in the formation of compound I, suggests that the presence of combined IPhNO lowers the affinity of haemoglobin for oxygen. Carbon monoxide, with its greater affinity for the pigment, is still able to combine, and is not displaced when IPhNO reacts with carboxyhaemoglobin.

IPhNHOH also reacts directly with carboxyhaemoglobin under conditions which preclude its oxidation to the nitroso compound; no alteration in absorption spectrum accompanies this process. Haemoglobin must combine similarly with the hydroxylamine in the presence of dithionite, for subsequent oxygenation yields an oxyhaemoglobin-like complex (VI, Fig. 2) and not compound I. For this reason it appears that oxyhaemoglobin converts added IPhNHOH into IPhNO before the formation of a pigment complex. It is likely that PhNHOH also combines directly with haemoglobin, for evidence to the contrary was based on the absence of spectroscopic change when it was added to reduced haemoglobin (Keilin & Hartree, 1943).

PhNHOH and IPhNHOH added to alkaline haematin produce the same sequence of colour changes. Since IPhNO forms directly the same end product as the iodophenylhydroxylamine, the reactions may be represented as follows:



SUMMARY

1. The reactions of haemoglobin and some of its derivatives with ^{131}I-labelled *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene have been followed spectroscopically and also by determining the combining ratio of the radioactive substance per atom of iron.

2. It has been shown that spectroscopic data alone are inadequate in the study of these reactions, for combination between pigment and iodo-compound may occur without accompanying change in absorption spectrum. Also, alteration in the absorption spectrum of a formed complex to that of a known blood pigment may be induced without loss of the combined radioactive material.

3. Iodophenylhydroxylamine combines directly with haemoglobin and carboxyhaemoglobin under conditions which preclude its oxidation to the nitroso compound.

4. A series of reactions follows the addition of the iodophenylhydroxylamine to oxyhaemoglobin. Three intermediates have been recognized and the final product is spectroscopically similar to methaemoglobin but retains one molecular equivalent of the iodo-compound. The interrelationships of these substances have been examined.

5. Carboxyhaemoglobin combines directly with iodophenylhydroxylamine or iodonitrosobenzene without loss of carbon monoxide or change in absorption spectrum. When oxyhaemoglobin reacts with iodonitrosobenzene, the oxygen is quantitatively displaced. It is suggested that the difference in behaviour between oxyhaemoglobin and carboxyhaemoglobin is due to the combined iodonitrosobenzene reducing the affinity of the pigment for oxygen; the much greater affinity of carbon monoxide, however, permits the retention of the latter.

6. It appears probable that phenylhydroxylamine and nitrosobenzene behave in a manner similar to their iodo-derivatives, so that the complexes formed with haemoglobin and its derivatives are not necessarily dissociated in subsequent reactions, as has been previously supposed.

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The Interaction of Dyes with Proteins on Paper with Special Reference to Paper Electrophoresis

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In attempts to develop quantitative techniques for the analysis of serum proteins separated by electrophoresis on filter paper, many workers have estimated the amount of dye taken up by the different protein fractions after staining. When comparisons have been made with analysis by the classical technique, quantitative differences have been noted (Cremer & Tiselius, 1950; Kunkel & Tiselius, 1951; Schneider, 1951; Grassmann & Hannig, 1952). Cremer & Tiselius used a factor of 1·6 to correct the difference on the grounds that the globulins had a lower binding power for bromophenol blue than had the albumin. This empirical factor was also used by Flynn & de Mayo (1951). Kunkel & Tiselius (1951) published a comparison of albumin and globulin uptake of bromophenol blue using a three-point plot. Grassmann & Hannig (1952) and Grassmann, Hannig & Knedel (1951), describing a procedure for the quantitative estimation of serum proteins using 'Amidoschwarz 10B' (naphthalein black 12B 200 of Imperial Chemical Industries Ltd.), pointed out the difference between albumin and γ-globulin in binding this dye. Martin (1952) stressed the gross differences in dye-binding capacity that existed between the various serum proteins and criticized the use of empirical correction factors derived from normal sera when studying abnormal sera. Slater & Kunkel (1953), though they give no data, clearly share this doubt about existing quantitative procedures, for they make no attempts at quantitative measurement of individual proteins in a study of urinary and serum proteins, and in their discussion write: 'it appears likely that quantitative procedures applied to filter-paper electrophoresis may be subject to considerable error....'.

The increasing use of filter-paper electrophoresis for separation of serum proteins, together with the very clearly expressed doubts by workers about the validity of the quantitative results, suggested that an attempt should be made to check some of the assumptions on which quantitative analysis is based.

MATERIALS AND METHODS

Dyes. Naphthalein black 12B 200, I.C.I. Ltd.; azocarmine B, from G. T. Gurr; bromophenol blue, and bromocresol green from British Drug Houses Ltd.

Absorption curves of the dyes in 0·01 N-NaOH were made in 1 cm. cells at room temp. in the Hilger Uvispec spectrophotometer standardized by hydrogen lines at 656·3, 486·1 and 434·0 mμ. Two such curves were plotted for each dye, one within 20 min. of the dye solution being made up, and the other after it had stood in a closed vessel in the dark for a day at room temp.

Qualitative analysis of the dyes used was carried out by descending filter-paper chromatography using *n*-butanol–acetic acid–water (5:1:4, v/v).

Serum proteins. Human albumin and γ-globulin fractions were prepared by the ether fractionating technique of Kekwick & Mackay (1954) and the composition was checked by electrophoresis according to the classical method of Tiselius. The proteins were taken up in 0·15 M-NaCl to give eleven albumin solutions ranging from 0·7 to 9·8% (w/v) and five γ-globulin solutions ranging from 0·3 to 5·0% (w/v). The protein concentrations were checked by estimating N (micro-Kjeldahl) using the factor 6·25 to convert to protein.

Paper electrophoresis was carried out in 0·06 M sodium diethylbarbiturate buffer (pH 8·6) (Flynn & de Mayo, 1951) by a method similar to that described by Franglen (1953). The paper used throughout this work was Whatman no. 1 as sold for chromatography.

Measurement of dye uptake by protein on filter paper. Each protein solution was pipetted on to dry sheets of filter paper by an Agla micrometer syringe (Trevan, 1925) as spots of 5, 10, 15, 20, 25, 30, 35 and 40 μl.; four spots at each volume were applied. The protein spots were dried at 105° for 30 min. They were then dyed for 10 min. in a solution of bromocresol green (0·2%, w/v) in 10% (v/v) ethanolic glacial acetic acid (Franglen, 1953). The pH of this solution was 3·2 by glass electrode standardized against 0·05 M potassium phthalate buffer (pH 4·00) (British Standards Institution (1950) 1647). The sheets were washed in 10% (v/v) aqueous acetic acid (pH 2·3) for 20 min., four changes of washing solution being used. At the end of this procedure, the background paper was clear of dye, and the sheets were blotted and allowed to dry at room temp.

The long and short axes of the spots were measured to the nearest mm. and from this the area was calculated to an accuracy of within 10%.

Constant areas of 3 × 3 cm. enclosing each dyed spot were cut from the sheets. The squares were eluted of dye by soaking in 5·0 ml. of 0·01 N-NaOH for at least 60 min. Recovery experiments showed that no less than 97% of the dye was eluted from the paper. Where necessary, the resulting blue solutions were diluted by adding known amounts of 0·01 N-NaOH to give $E^{613\,m\mu}_{1\,cm.}$ not greater than 0·500. The $E^{613\,m\mu}_{1\,cm.}$ of each solution was determined by means of a Unicam spectrophotometer S.P. 350. Over the range 0·000–0·500 $E^{613\,m\mu}_{1\,cm.}$ these solutions of bromocresol green showed approximately 5% deviation from Beer's Law, but

by reference to a calibration curve from sixteen different solutions of known composition, this deviation was allowed for when the bromocresol green concentration was calculated from the $E^{613\,m\mu}_{1\,cm.}$.

Calculation of results. For each concentration of protein, two plots were made. These consisted of the weight of dye eluted from each spot plotted against: (1), the weight of protein applied to the paper; (2), the area of each protein spot. These plots approximated closely to straight lines passing near or through the origin, the slopes being respectively equal to: (1), the weight of dye bound to unit weight of protein; (2), the weight of dye eluted from unit area of protein spot. To get as accurate an estimation as possible of the slope, straight lines were fitted on to the plots by the method of least squares.

EXPERIMENTAL

The absorption curves in 0·01 N-NaOH of the four dyes commonly used for staining protein on filter paper are shown in Fig. 1 (*a–d*). The absorption maximum of bromocresol green is at 613 mμ., of bromophenol blue 595 mμ., of fresh naphthalein black 610 mμ., and of azocarmine B 520 mμ. It will be seen that at these wavelengths, the order of decrease of $E^{1\%}_{1\,cm.}$ is bromophenol blue, bromocresol green, naphthalein black and azocarmine B, the maximum absorption of a 1% solution of azocarmine B being less than one-fifth of that of a 1% solution of bromophenol blue. The molecular extinctions (ϵ) for bromophenol blue and bromocresol green, the two dyes for which this figure could be expressed, were $6{\cdot}6 \times 10^4$ and $4{\cdot}57 \times 10^4$ respectively. This figure also shows that the absorption of both naphthalein black and bromophenol blue diminish appreciably on standing; a comparison of the rate of fading of bromophenol blue with that of bromocresol green is shown in greater detail in Fig. 2. Bromophenol blue is more stable in 0·01 N-Na_2CO_3, as pointed out by J. Hardwicke (personal communication).

Chromatographic analysis of samples of azocarmine B used in this work demonstrated that the dye contained at least six components, as shown in Fig. 3. Similar analysis of bromocresol green demonstrated one sharply defined component. Analysis of bromophenol blue suggested trace impurities.

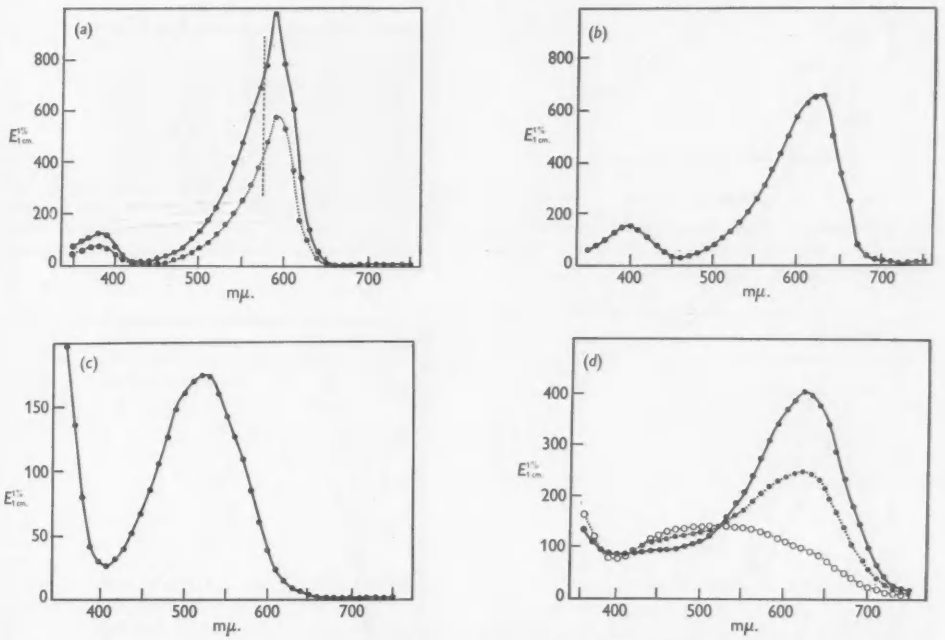

Fig. 1. (*a*) Absorption curves of bromophenol blue (1%, w/v, in 0·01 N-NaOH). ●—●, within 20 min. of solution; ●- - -●, 26 hr. after solution. Vertical dashes represent the wavelength used for measurements of bromophenol blue by Kunkel & Tiselius (1951). (*b*) Absorption curve of bromocresol green (1%, w/v, in 0·01 N-NaOH) within 20 min. and at 26 hr. after solution. (*c*) Absorption curve of azocarmine B (1%, w/v, in 0·01 N-NaOH) within 20 min. and at 26 hr. after solution. (*d*) Absorption curve of naphthalein black 12 B 200 (1%, w/v, in 0·01 N-NaOH). ●—●, within 20 min. of solution; ●- - -●, 26 hr. after solution; ○---○, 54 hr. after solution.

Fig. 4 shows the result of a comparison of bromocresol green uptake of albumin with that of γ-globulin presented in terms of (*a*) g. dye uptake/g. protein against concentration of protein applied to the paper, and (*b*) g. dye uptake/mm.^{2} protein spot area against concentration of protein solution. It will be seen that the curves obtained from the two proteins are neither linear nor superimposed. Fig. 5 shows the data presented differently. Here the ratio of dye uptake of γ-globulin to dye uptake of albumin for a standard area (Fig. 5*a*) and a standard weight (Fig. 5*b*) are plotted against γ-globulin concentration, plots being shown for a series of concentrations of albumin. The results emphasize the impossibility of obtaining reasonably accurate analysis of a two-component system by dye-uptake techniques.

This experiment does not mirror precisely the conditions of analysis of mixtures in practice. Solutions were prepared therefore containing vary-

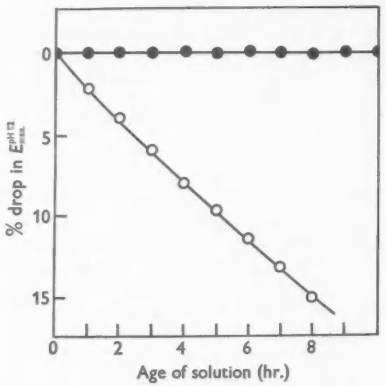

Fig. 2. Comparison of the alteration of extinction with time (1%, w/v, dye in 0·01 N-NaOH). ○—○, bromophenol blue; ●—●, bromocresol green.

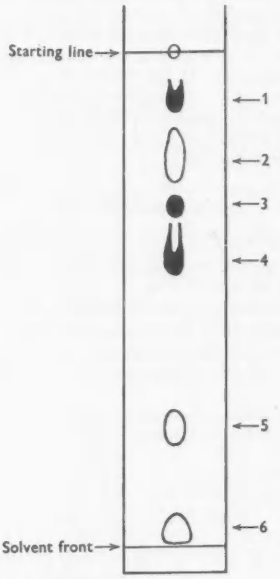

Fig. 3. Drawing of filter-paper chromatogram of azocarmine B run in aqueous butanol–acetic acid mixture. Black spots represent areas of red fluorescence under light of 253·7 mμ.; white spots represent areas of orange fluorescence.

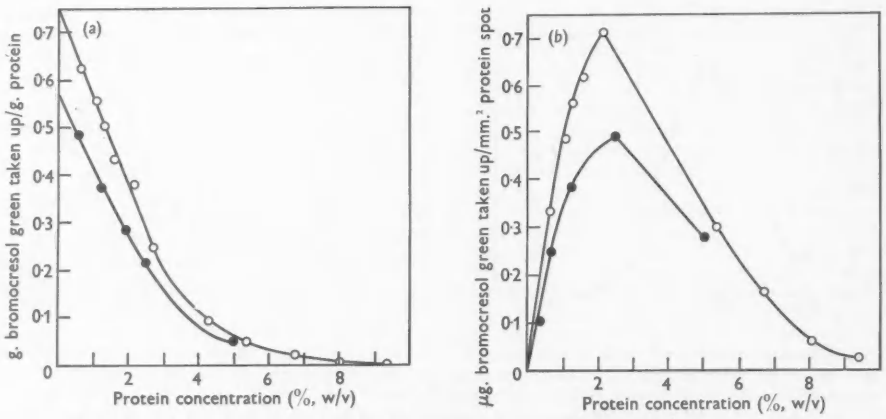

Fig. 4. Uptake of bromocresol green by protein fractions on (*a*) weight and (*b*) area basis. ○—○, albumin; ●—●, γ-globulin.

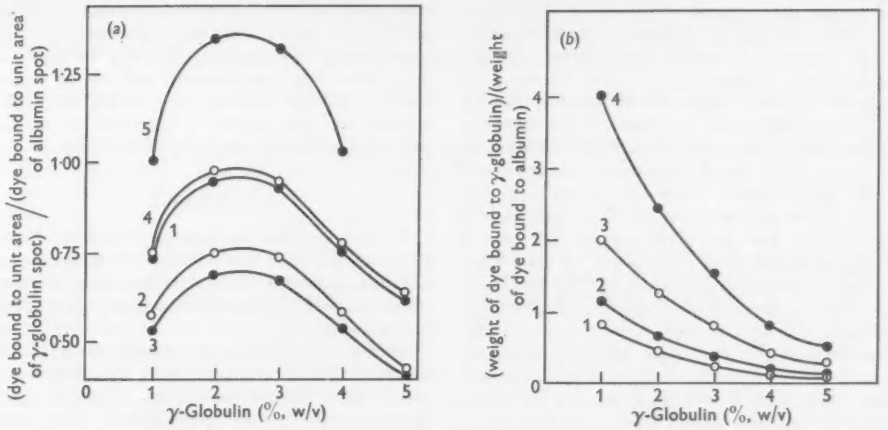

Fig. 5. (*a*). Ratio of bromocresol green bound by unit area of γ-globulin to bromocresol green bound by unit area of albumin spot plotted against concentration of γ-globulin at albumin concentrations (%, w/v) shown beside each curve. (*b*) Ratio of bromocresol green bound by unit weight of γ-globulin to bromocresol green bound by unit weight of albumin plotted against concentration of γ-globulin at albumin concentrations (%, w/v) shown beside each curve.

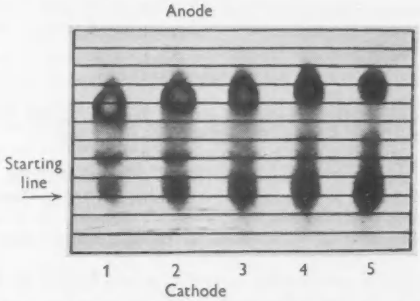

Fig. 6. Filter-paper electrophoreses of 10 μl. spots of mixtures of albumin and γ-globulin run in parallel in 0·06 M sodium diethylbarbiturate buffer (pH 8·6) for 12 hr. at a potential gradient of 3·8 v/cm. and dyed with bromocresol green. Grid lines 1 cm. apart.

	Albumin (g./100 ml.)	γ-Globulin (g./100 ml.)
1	9·6	1·0
2	7·7	2·0
3	4·8	3·0
4	3·8	4·0
5	2·0	5·0

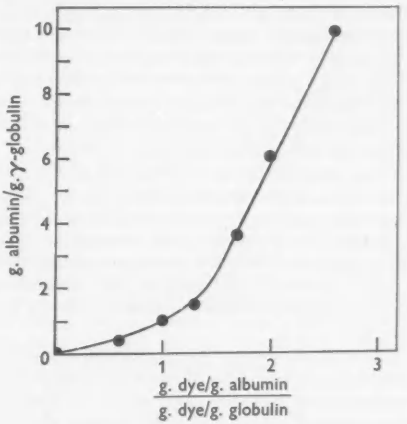

Fig. 7. Plot of true ratios of albumin to γ-globulin against ratios calculated directly from bromocresol green uptake (see Fig. 5 and text).

ing proportions of albumin and γ-globulin. 10 μl. of each of these solutions were then subjected to simultaneous electrophoresis on paper. Fig. 6 shows a photograph of one such run, and Fig. 7 shows graphically the plotting of the ratio of albumin to γ-globulin arrived at by dye elution plotted against the true ratio. It will be seen that a linear relation is achieved at ratios which only occur rarely in human serum-protein analyses.

DISCUSSION

Previous writers (see Tiselius & Flodin, 1953) have observed the discrepancy between quantitative analysis using staining techniques on paper and the classical methods in solution. The majority have sought to circumvent this discrepancy by the introduction of empirical factors based on the deviations demonstrated in normal sera with the tacit assumption that these factors are adequate when the ratios of the components are altered, as

they are in many disease states. This assumption demands, if nothing else, a direct linear relationship between individual protein concentration and dye uptake. Some attempt at a more fundamental approach has been made by Kunkel & Tiselius (1951). They have published plots of dye intensity against protein concentration, contrasting albumin and γ-globulin stained with bromophenol blue, though, unfortunately, they did not mention any correction for the fading of bromophenol blue in 0·01 N-NaOH, nor did they explain the use of 575 mμ. as the selected wavelength for estimation after extraction, when it appears that the maximum absorption for bromophenol blue is nearer 600 mμ. Bromocresol green, a close analogue of bromophenol blue, is free from the hazard of fading exhibited by both naphthalein black 12B and bromophenol blue and is homogeneous on chromatography in aqueous butanol–acetic acid mixture unlike azocarmine B. It would seem therefore to be the dye of choice if dye-elution or scanning techniques are to be used.

There is, however, another and more fundamental reason for doubting the validity of the techniques than the unfortunate choice of relatively unsuitable dyes. The plot of the dye bound to a protein against the concentration of that protein is not linear. Moreover, the characteristics of the relation of dye binding to protein concentration differ from one protein to another. Further, if two protein solutions of known concentrations are mixed in predetermined proportions and are then separated by paper electrophoresis, the proportions of these two components calculated by dye-binding techniques fail to tally with the predetermined ratios. Moreover, when a series of predetermined mixtures is examined, there is no consistent relationship which enables a correction factor to be applied.

If this be so when a two-component system is studied, it does not seem reasonable to suppose that a system of four components or more will be capable of more exact analysis. It would seem, therefore, that quantitative data based on the use of dyes with proteins separated by electrophoresis on paper needs drastic reassessment. Though the method in its present form has considerable use as a qualitative tool, the present data throw very grave doubts on the value of quantitative analyses produced by existing staining techniques.

SUMMARY

1. The spectral absorption characteristics of bromophenol blue, bromocresol green, azocarmine B, and naphthalein black 12B have been examined. Azocarmine B is heterogeneous on chromatography on filter paper.

2. The dye-binding characteristics of human albumin and human γ-globulin with bromocresol green have been studied individually and in mixtures of known composition. The results suggest that existing quantitative methods of analysis by paper-strip electrophoresis require reassessment.

We wish to acknowledge the financial help given by the Research Fund of St George's Hospital and to thank Dr M. Mackay of the Lister Institute for supplies of albumin and γ-globulin.

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Studies on the Biosynthesis of Riboflavin

NITROGEN METABOLISM AND FLAVINOGENESIS IN *EREMOTHECIUM ASHBYII*

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Many fungi are known to synthesize riboflavin in small amounts (e.g. Peltier & Borchers, 1947, list 240 flavin-producing species), but there are three which synthesize it and excrete it into the culture medium in very large amounts. These are *Candida* spp. (Burkholder, 1943; Tanner, Vojnovich & van Lanen, 1949), *Eremothecium ashbyii* (Guillermond, Fontaine & Raffi, 1935) and *Ashbya gossypii* (Wickerham, Flickinger & Johnston, 1946).

Although many patents have been filed covering the commercial production of riboflavin using these organisms (see a review by Pridham, 1952, for full details), the observations recorded have in general been only of limited value in furthering knowledge of the basic mechanisms concerned in this biosynthesis. The main reason for this is that the cheaply available materials found to stimulate riboflavin production are generally such biochemically complex products as molasses, fish meal, hide scraps, dried blood, etc.

A considerable amount of work has been carried out in an attempt to culture *E. ashbyii* on a fully defined medium. (Schopfer, 1944; Schopfer & Guilloud, 1945*a*–*c*; Dulaney & Grutter, 1950; Maclaren, 1952; Yaw, 1952; Hickey, 1953), but a survey of the literature makes it clear that the problem has not yet been completely solved. Our preliminary experiments confirmed this conclusion; it was found, however, that satisfactory and reproducible growth and riboflavin synthesis could be obtained using small amounts of peptone in an otherwise synthetic culture medium. Using this type of basal medium, an investigation has been carried out on the effect of various nitrogen-containing compounds on riboflavin synthesis by *E. ashbyii*. A preliminary report of some of these findings has already been made (Goodwin & Pendlington, 1954).

EXPERIMENTAL

Cultures. The culture used throughout this investigation was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland. It was maintained on malt agar slopes and subcultured every 7–10 days.

Media. The basal medium used contained per l.: glucose, 10 g.; $CaCl_2$, 0·2 g.; $MgSO_4$, $7H_2O$, 1·0 g.; NaCl, 0·2 g.; KH_2PO_4, 2·0 g.; biotin 0·04 mg.; thiamine, 0·1 mg.; inositol, 0·2 g. To this was added varying amounts of bacteriological peptone (Evans) and the nitrogen compounds under test. The pH was adjusted to 5·8. Sterilization was carried out by autoclaving at 15 lb./sq.in. for 15 min. Although the peptone would probably contain sufficient quantities of inositol, biotin and thiamine, it was considered advisable to add always the optimum amounts advised by Schopfer (1944). This would eliminate possible variations in the vitamin content of different batches of peptone and also the difference in vitamin levels when various concns. of peptone were being compared.

All the amino acids used except tyrosine, phenylalanine and tryptophan and all the purines and pyrimidines were obtained from L. Light & Co. Ltd., Colnbrook, Bucks; tyrosine, phenylalanine and tryoptophan were obtained from British Drug Houses Ltd., London; 1:2-dimethyl-4-amino-5-(D-1′-ribitylamino)benzene and 1:2-dimethyl-5-(D-1′-ribitylamino)benzene were the gift of Dr E. S. Holdsworth and 4:5-dimethylbenziminazole the gift of Dr V. Petrow.

Cultural conditions. The media (15 ml.) were dispensed in Erlenmeyer flasks (50 ml.), inoculated and incubated in a water-jacketed incubator in the dark at 28° without shaking. The patent literature makes it clear that riboflavin synthesis is stimulated considerably in shake cultures. It was not possible to shake the cultures in the present investigation, but this is no drawback providing the conditions are always standardized. Each flask was inoculated with three drops of a spore suspension drawn from a 2-day-old culture grown on the basal medium plus 0·4% (w/v) peptone. This procedure was adopted following the observation of Tanner *et al.* (1949) that, with *Ashbya gossypii*, best yields of riboflavin were obtained when the inoculum vol. was about 0·5% of the total vol. of the medium and when it was obtained from young cultures. This point was not tested in *E. ashybii*, but the procedure just described was found to be extremely satisfactory and to yield very reproducible results.

Analytical procedures. Three replicate flasks were examined in each experiment. The major part of the medium and mycelium was transferred to a thin, 15 ml. tared centrifuge tube and centrifuged in a small bench centrifuge for 5 min. A portion of the supernatant was taken for riboflavin determination and the remainder discarded; any mycelium remaining in the culture flask was transferred quantitatively to the centrifuge tube which was again spun for 5 min. The residual medium was decanted off and the mycelium twice washed thoroughly with distilled water, followed each time by centrifuging. The washed mycelium was then dried at 80° for 24 hr.

Riboflavin in the medium was determined spectrophotometrically by measuring the E value of the centrifuged medium at 445 mμ., the wavelength of max. absorption for

riboflavin in the visible region of the spectrum. By comparing this value with the $E^{1\%}_{1\,cm.}$ (445 mμ.) value for pure riboflavin (301; Adamson, 1948), the amount of riboflavin in the medium could easily be calculated. This procedure was justified for a number of reasons: (*a*) the starting medium exhibited almost negligible absorption at 445 mμ., (*b*) the absorption spectra of different specimens of riboflavin-containing media were not significantly different from that of pure riboflavin, and (*c*) paper chromatography of the medium according to the method of Crammer (1948) for riboflavin and its derivatives showed that the pigment was almost entirely riboflavin; apart from riboflavin there only existed minute traces of a spot with blue fluorescence in u.v. light with an R_F value very close to that given by Crammer (1948) for riboflavin phosphate. The riboflavin in the mycelium was extracted by adding 10 ml. of HCl to the washed residue in the centrifuge tube and autoclaving at 15 lb./sq.in. for 15 min. After cooling, the supernatant, was made up to an appropriate volume (usually 15 ml.) and the riboflavin determined spectrophotometrically. The residual mycelium was completely colourless.

RESULTS

Reproducibility of results

Table 1 shows twelve triplicate values obtained for riboflavin synthesis in the basal medium containing 5 mg. peptone N/100 ml. These results were obtained over a period of 18 months and show that whilst reproducibility between flasks in any one experiment is usually excellent, the amounts of riboflavin produced under apparently identical cultural conditions at different times can vary considerably. The reason for these variations is not known, but they emphasize the need for adequate controls. The 'experimental' flasks showed the same range of variations. Occasionally (about 1 in 200 flasks), an aberrant flask is obtained, in which riboflavin synthesis is very much greater than expected, but growth is very much reduced.

Although most of the stimulatory effects dealt with in this paper are large, generally at least 50% above normal, it is obvious from Table 1 that under optimum conditions it would be possible to demonstrate significant stimulations of the order of 10–20% above normal.

The effect of age on growth and riboflavin synthesis

A typical time curve for growth and flavinogenesis by *E. ashbyii* cultured on the basal medium containing 0·24% (w/v) peptone (Fig. 1), shows that growth was complete after 5 days but that riboflavin production, increasing at almost the same rate as growth during the first 5 days after inoculation, continued until about the seventh or eighth day. Thereafter, net production of riboflavin was almost nil. Following these observations, it was decided that in subsequent experiments only 5- and/or 9- to 10-day cultures would be examined.

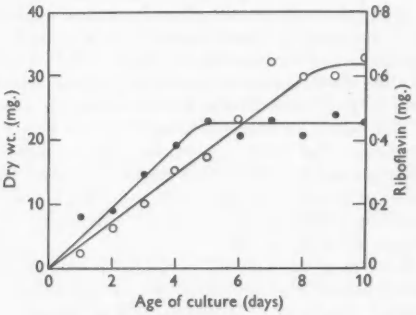


Fig. 1. Dry wt. and riboflavin production by *E. ashbyii* cultured on basal medium containing 0·24% (w/v) peptone. Temp. 28°. Amounts per 15 ml. medium in 50 ml. conical flasks. ●—●, dry wt.; o—o, riboflavin.

Table 1. *Reproducibility of growth and riboflavin production by* E. ashbyii

Triplicate analyses made during different experiments; production in 15 ml. medium in 50 ml. conical flasks. Incubation time 5 days; temp. 25°. Basal medium +5 mg. peptone N/100 ml.

Expt. no.	Riboflavin (μg./15 ml.) Individual values			Mean	Dry wt. of mycelium (mg.) Individual values			Mean
1	145	159	152	152	9·8	9·7	9·7	9·7
2	122	177	170	156	11·4	12·5	11·1	11·7
3	185	175	158	173	10·5	13·3	11·7	11·8
4	185	214	178	192	8·2	6·9	7·0	7·4
5	250	305	249	268	13·1	11·3	11·3	11·9
6	180	188	188	185	13·6	12·4	14·5	13·5
7	145	150	140	145	10·7	10·0	8·4	9·7
8	106	102	111	106	12·8	12·1	12·3	12·4
9	167	176	193	175	—	10·7	11·7	11·2
10	146	130	136	137	8·6	10·5	6·2	8·4
11	182	208	178	189	11·8	11·0	8·4	10·4
12	254	246	238	246	7·1	7·0	7·0	7·0

The effect of the concentration of peptone

The effect of varying the concentration of peptone was examined in the presence and absence of 0·1% (w/v) glycine. Table 2 shows that the presence of glycine had no effect on the action of peptone on riboflavin production; there does appear to have been in this case a slight stimulatory effect on growth, although this was not confirmed in other experiments (see, for example, Table 4).

Comparing Fig. 1 and Table 2, it will be seen that the observations recorded in Fig. 1, that growth but not flavinogenesis is complete after 5 days' incubation, have been confirmed.

Table 1 also indicates that the optimum concentration of peptone for riboflavin synthesis is about 0·24%, if the amount per unit weight of mycelium but not the total yield is the criterion; this is most marked in 10-day cultures. Further experiments (Table 3) showed, however, that the spread is rather wide, and that the highest concentrations of riboflavin are produced over a peptone concentration range of 0·16–0·40% (w/v).

Table 2. *Growth and riboflavin synthesis by* E. ashbyii *in presence and absence of glycine and in presence of varying amounts of peptone*

Dry wt. and riboflavin per 15 ml. medium in 50 ml. conical flasks; basal medium described in Experimental section; temp. 28°.

	Five-day cultures			Ten-day cultures		
		Riboflavin			Riboflavin	
Concn. of peptone* % (w/v)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)
			Medium without glycine			
1·92	32·8	904	2·76	42·6	1499	3·52
0·96	33·4	768	2·32	33·7	2052	6·08
0·48	30·2	715	2·41	27·9	1856	6·65
0·24	21·1	442	2·25	21·0	1644	7·82
0·12	16·4	465	2·82	13·0	1071	8·22
0·06	11·0	281	2·99	11·8	717	6·07
0·03	8·5	166	1·95	9·8	405	4·03
			Medium containing glycine 1·0% (w/v)			
1·92	41·4	947	2·34	54·7	1614	2·95
0·96	40·1	799	2·00	38·4	1884	4·90
0·48	29·0	723	2·49	26·4	1640	6·22
0·24	26·1	656	2·51	20·7	1385	7·99
0·12	20·8	491	2·39	18·5	1021	5·55
0·06	16·0	282	1·84	16·1	692	4·31
0·03	9·1	124	1·36	11·3	276	2·41

* Peptone contains 12·5% N.

Table 3. *Growth and riboflavin synthesis by* E. ashbyii *in presence of varying peptone concentrations*

Dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; basal medium supplemented with varying amounts of peptone.

	Five-day cultures			Nine-day cultures		
		Riboflavin			Riboflavin	
Concn. of peptone* % (w/v)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)
0·040	19·0	185	1·01	10·8	345	3·19
0·080	21·2	340	1·45	15·6	684	4·38
0·160	25·0	610	2·42	19·5	1052	5·35
0·240	27·2	686	2·52	25·5	1333	5·23
0·320	29·5	725	2·45	29·9	1574	5·26
0·400	30·6	717	2·35	30·3	1581	5·22
0·480	31·3	725	2·32	32·8	1563	4·76

* The peptone used contained 12·5% N.

Effect of various amino acids on flavinogenesis

Two control media were used: *A* consisted of the basal medium plus peptone (≡0·005 %, w/v, N); which gave a reasonable growth (about 10 mg. dry wt. per 15 ml.) but only a comparatively small production of riboflavin. Medium *B* consisted of the basal medium plus 0·025 % (w/v) peptone N. This gave about twice the dry weight produced by *A* and more than twice the amount of riboflavin. By adding various N sources to medium *A* in amounts calculated to bring the total N content of the medium to that of *B*, the ability of the N sources to stimulate the synthesis of riboflavin could be assessed and compared with that of peptone. Table 4 gives the results obtained when most of the known amino acids except tyrosine and tryptophan (see later) were examined in this way. It will be seen that most of these amino acids are incapable of stimulating either growth or flavinogenesis, whilst L-cysteine inhibited growth completely. Two amino acids, however, DL-serine and DL-threonine, specifically stimulated riboflavin production. L-Asparagine, L-aspartic acid and L-glutamic acid stimulated both growth and riboflavin production equally, as evidenced by the same concentrations of riboflavin in these cultures as in the *B* controls. They could not, therefore, be considered to have a specific effect on riboflavin. A few amino acids (e.g. L-cystine) tended to inhibit growth slightly without affecting riboflavin synthesis. This resulted in a somewhat higher riboflavin concentration than in the control medium, but as the effect was slight by comparison with that of DL-threonine and DL-serine and, more important, because the amount of riboflavin was never greater than that in the controls, these amino acids were not examined further.

Of the two specifically stimulating amino acids, threonine was examined in more detail and it was shown (Fig. 2) that maximal stimulation was achieved with 0·5 mg. L-threonine N/100 ml. Further experiments showed that D-threonine was inactive but in no way inhibitory, whilst, as might have been expected, DL-threonine was (below the plateau level) one half as active as the L isomer. In the presence of 25 mg. peptone N/100 ml., the effect of L-threonine is almost obliterated, presumably because sufficient is present in the extra peptone.

The two common amino acids not included in Table 4 are L-tyrosine and L-tryptophan. They were not sufficiently soluble at the pH of the medium to be examined at a level of 20 mg. N/100 ml. They were, therefore, examined at lower concentrations

Table 4. *Growth and riboflavin synthesis by* E. ashbyii *in presence of various amino acids*

Dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; temp. 28°; media *A* and *B* contain the basal medium + 5 mg./100 ml. and 25 mg./100 ml. of peptone N respectively: other media contain 5 mg. peptone N/100 ml. plus an amino acid at a concentration equivalent to 20 mg. N/100 ml.

	Five-day cultures			Ten-day cultures		
		Riboflavin			Riboflavin	
Medium	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)
Control *A*	13·7	230	1·68	14·0	395	2·82
L-Alanine	7·9	143	1·82	9·2	365	3·38
DL-α-Amino butyric acid	9·6	118	1·51	7·5	333	4·44
L-Arginine	13·6	176	1·29	16·4	443	2·71
L-Asparagine	27·2	506	1·95	24·5	915	3·73
L-Aspartic acid	20·5	593	2·90	26·3	1025	3·90
DL-Citrulline	14·5	253	1·75	13·0	540	4·15
L-Cysteine		No growth			No growth	
L-Cystine	11·1	283	2·58	9·7	348	3·27
L-Glutamic acid	17·5	547	3·12	19·0	842	4·43
Glycine	13·7	141	1·07	17·7	360	2·05
L-Hydroxyproline	13·4	213	1·59	9·0	337	3·74
DL-Histidine	16·9	179	1·06	—	—	—
DL-Isoleucine	15·6	166	1·06	16·7	235	1·45
L-Leucine	15·2	190	1·26	18·3	425	2·33
L-Lysine	13·1	192	1·43	13·6	368	2·71
DL-Methionine	14·8	135	0·91	16·2	194	1·19
DL-Ornithine	15·8	152	0·96	—	—	—
L-Phenylalanine	10·9	114	1·30	13·4	288	2·15
L-Proline	16·4	191	1·16	20·7	328	1·59
DL-Serine	14·5	343	2·32	12·3	973	7·80
DL-Threonine	10·9	428	3·93	13·9	865	6·23
L-Valine	18·4	176	0·95	19·5	359	1·84
Control *B*	22·6	473	2·11	24·3	985	4·06

Table 5. *Growth and riboflavin synthesis by* E. ashbyii *in the presence of small amounts of* L-*tyrosine*

Media *A* and *B* are the basal medium plus 5 and 13 mg. peptone N/100 ml., respectively; dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; temp. 28°.

	Five-day cultures			Ten-day cultures		
		Riboflavin			Riboflavin	
Concn. of tyrosine added (mg. N/100 ml.)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control *A*)	10·8	285	2·64	8·7	547	6·29
0·5	8·3	262	3·16	9·3	492	5·32
1·0	10·7	282	2·64	8·5	529	6·24
2·0	9·7	370	3·81	9·0	539	6·00
4·0	10·4	393	3·78	9·0	624	6·99
8·0	7·2	465	6·42	12·8	741	5·67
0 (control *B*)	15·1	469	3·10	15·1	1036	6·90

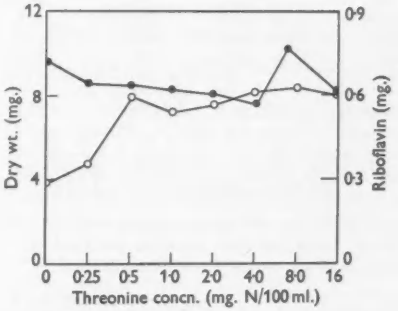


Fig. 2. The effect of the addition of varying amounts of L-threonine to the basal medium plus 0·005% (w/v) peptone N, on dry wt. and riboflavin production. Cultural conditions are the same as described for Fig. 1. •—•, dry wt.; o—o, riboflavin.

Table 6. *Growth and riboflavin synthesis by* E. ashbyii *in presence of small amounts of* DL-*phenylalanine*

Media *A* and *B* are the basal medium plus 5 and 13 mg. peptone N/100 ml., respectively. Dry wt. and riboflavin production per 15 ml. medium in 50 ml. conical flasks; 5 day cultures; temp. 28°.

		Riboflavin	
Concn. of phenylalanine added (mg. N/100 ml.)	Dry wt. (mg.)	Total amount (μg.)	Amount (g./100 g. dry mycelium)
0 (control *A*)	11·1	347	3·19
0·5	8·9	327	3·71
1·0	8·7	289	3·32
2·0	9·2	278	3·01
4·0	9·8	320	3·28
8·0	7·4	271	3·66
0 (control *B*)	18·1	574	3·35

(0·5–8·0 mg. N/100 ml.) and it was found that DL-tryptophan was without effect on flavinogenesis, although growth was inhibited slightly; at the highest concentration flavinogenesis as well as growth appeared to be inhibited. L-Tyrosine, on the other hand, stimulated riboflavin synthesis, but was much less effective than threonine (Table 5). The first unequivocal response was obtained with a level of 4·0 mg. N/100 ml., whilst with threonine maximal response was obtained with 0·5 mg. N/100 ml. (Fig. 2).

The possibility existed that the weak effect of tyrosine was due to an impurity in the sample used. Paper chromatography revealed only one spot (tyrosine) and no other amino acid was detected. The presence of a trace impurity in tyrosine appears to be unlikely, for such an impurity might be expected to occur also in other amino acids, e.g. L-phenylalanine which is probably isolated from a similar source. DL-Phenylalanine was examined in more detail but with entirely negative results (Table 6).

Paper chromatography (Crammer, 1948) of the media in which riboflavin synthesis had been stimulated showed that in all cases the flavin produced was chromatographically indistinguishable from riboflavin.

Effect of purines and pyrimidines

Following the observations of Maclaren (1952) that certain purines stimulated flavinogenesis by *E. ashbyii*, a number of purines and pyrimidines and their derivatives were tested under the same conditions as were the amino acids. Table 7 shows that xanthine and adenine (as well as adenosine) specifically stimulated riboflavin considerably whilst the pyrimidines and their derivatives had no such effect. There is some inhibition of both growth

and riboflavin synthesis with thiouracil but no specific effect on riboflavin; alloxan is very much more inhibitory than thiouracil.

More detailed examination of adenine and xanthine (Tables 8 and 9) show that xanthine tends to be somewhat more effective than adenine but that, in contrast to the observations with the flavinogenic amino acids, the stimulation continues to increase up to the highest levels examined. It will be noticed that with adenine there was no stimulation of growth even with the highest concentrations, but that with xanthine there appeared to be some growth stimulation at high concentrations.

Table 7. *The effect of purines and pyrimidines on riboflavin production by* E. ashbyii

Controls *A* and *B* contain the basal medium + 5 and 25 mg. peptone N/100 ml. respectively. The various purines and pyrimidines were added to *A* in amounts equivalent to 20 mg. N/100 ml. Six-day cultures; temp. 28°. Amounts are those produced by 15 ml. medium in 50 ml. flasks.

Culture medium	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
Control *A*	13·0	290	2·23
A + adenine	11·4	572	5·02
A + adenosine	12·7	644	5·06
A + xanthine	13·7	788	5·76
A + thymine	10·8	187	1·73
A + cytosine	11·5	301	2·63
A + uracil	12·1	228	1·88
A + thiouracil	7·0	169	2·46
A + alloxan	1·4	32	2·27
Control *B*	22·8	685	3·00

Table 8. *The effect of varying concentrations of adenine on riboflavin synthesis by* E. ashbyii

Media *A* and *B* contain the basal medium + 5 and 45 mg. peptone N/100 ml. respectively; 5-day culture; temp. 28°. Amounts produced in 15 ml. medium in 50 ml. conical flasks.

Concn. of adenine added (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
0 (control *A*)	11·3	215	1·90
0·25	10·4	223	2·14
0·50	11·3	304	2·69
1·0	10·6	344	3·25
2·0	11·8	362	3·07
4·0	11·4	422	3·70
8·0	10·6	442	4·17
16·0	12·0	495	4·13
20·0	12·6	562	4·46
30·0	12·8	597	4·66
40·0	11·3	720	6·37
0 (control *B*)	21·5	576	2·68

It was also found that even in the presence of 25 mg. peptone N/100 ml. small amounts of the purines stimulated riboflavin synthesis. For example, in one experiment the addition of 8 mg. adenine N/100 ml. increased flavinogenesis by 36 %.

Table 9. *The effect of varying concentrations of xanthine on riboflavin synthesis by* E. ashbyii

Experimental conditions as outlined in Table 8.

Concn. of xanthine added (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
0 (control *A*)	15·9	366	2·29
0·25	13·7	401	2·92
0·50	13·6	437	3·22
1·0	15·0	512	3·40
2·0	15·9	708	4·42
4·0	14·4	699	4·86
8·0	18·1	722	3·98
16·0	17·8	962	5·38
20·0	21·1	995	4·84
30·0	18·9	1137	6·04
40·0	(Not examined—xanthine not sufficiently soluble)		
0 (control *B*)	30·6	770	2·52

Table 10. *The effect of uracil on growth and riboflavin synthesis by* E. ashbyii

The control is the basal medium + 0·005 g. peptone N/100 ml. 5-day cultures; amounts produced in 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Amount of uracil added to medium (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
0 (control)	10·8	262	2·43
50	8·0	92	1·03
150	10·2	130	1·28
200	11·6	121	1·04
250	9·5	161	1·69

Table 11. *The effect of uracil on the adenine-stimulated synthesis of riboflavin by* E. ashbyii

Control medium is basal medium + 0·005 % (w/v) peptone N; 5-day cultures; amounts produced by 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Amounts (mg. N/100 ml.) added to basal medium: Adenine	Amounts (mg. N/100 ml.) added to basal medium: Uracil	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
0	0	9·7	145	1·50
4	0	9·6	252	2·71
4	2	12·6	280	2·2
4	4	10·8	254	2·4
4	8	11·7	229	2·0
4	16	11·2	210	1·9

The results given in Table 7 indicated that uracil had only a doubtful inhibitory effect on riboflavin synthesis when tested at a low concentration compared with that used by Maclaren (1952), who obtained marked inhibition. Uracil was accordingly tested under our conditions at the levels employed by Maclaren. It will be seen (Table 10) that at concentrations of 50 mg. N/100 ml. and above very little effect on growth was observed but that flavinogenesis was inhibited to the extent of about 50%. If, as Maclaren suggested, uracil exerts its inhibitory action by blocking the incorporation of a purine into the riboflavin molecule, then this effect should be most marked under conditions where purines would normally be stimulating riboflavin synthesis. Table 11 shows that under these conditions using adenine as the purine, there was only very slight inhibition even when the uracil concentration was 4 times that of adenine.

Effect of derivatives of 4:5-dimethyl-o-phenylenediamine

Other nitrogeneous compounds which might be precursors of riboflavin are 5:6-dimethylbenziminazole and 1:2-dimethyl-4-amino-5-(D-1′-ribitylamino)benzene. These, together with 1:2-dimethyl-5-(D-1′-ribitylamino)benzene, have been examined in the same way as the previous compounds at a level equivalent to the level of adenine (4 mg. N/100 ml.) used in previous experiments. The results of one of these experiments are recorded in Table 12. No stimulation was observed and in fact there was in two cases slight but definite inhibition. This inhibition has been confirmed in other experiments (see, for example, Table 14).

Miscellaneous nitrogen compounds

A number of other nitrogeneous compounds have also been examined and the results of one such experiment are recorded in Table 13. It will be seen that glucosamine considerably inhibits both growth and riboflavin synthesis equally, whilst $(NH_4)_2SO_4$ is without obvious effect. Urea is interesting in that in all experiments at the higher level it has proved non-stimulatory for riboflavin; at the lower level, on the other hand, it is occasionally slightly stimulatory, as in the experiment recorded in Table 13. The reason for this spasmodic stimulation is not known.

Combined effect of various nitrogen sources

The effect on riboflavin synthesis of the addition of other nitrogeneous substances to a medium containing L-threonine at its optimum level has been examined. In the results set out in Fig. 3, riboflavin production is recorded as the percentage stimulation above the control level. It was not considered necessary to record the dry weights of the mycelia, although they were measured, because, as shown earlier, none of the substances examined

Table 12. *The effect of* 4:5-*dimethyl-*o*-phenylenediamine derivatives on riboflavin synthesis by* E. ashbyii

Control medium is basal medium + 0·005% (w/v) peptone N; 5-day cultures; amounts produced by 15 ml. medium in 50 ml. conical flasks; temp. 28°.

Compound tested	Amount added* (mg./100 ml.)	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
0 (control)	0	10·4	175	1·68
5:6-Dimethylbenziminazole	7·4	9·8	126	1·28
1:2-Dimethyl-4-amino-5-(D-1′-ribitylamino)benzene	3·9	10·1	148	1·46
1:2-Dimethyl-5-(D-1′-ribitylamino)benzene	4·1	10·2	178	1·74

* These amounts are the molecular equivalents of 7·7 mg. adenine, which contains 4·0 mg. N.

Table 13. *The effect of some miscellaneous nitrogenous compounds on riboflavin synthesis by* E. ashbyii

Control media *A* and *B* contain the basal constituents + 5 and 25 mg. peptone N/100 ml. respectively. Amounts produced by 15 ml. of medium in 50 ml. conical flasks; temp. 28°; 5-day cultures.

Constituent added to medium	Concentration of constituent (mg. N/100 ml.)	Dry wt. (mg.)	Riboflavin: Total amount (μg.)	Riboflavin: Amount (g./100 g. dry mycelium)
0 (control *A*)	0	11·3	213	1·89
Glucosamine	20	4·8	70	1·52
Urea	5	10·0	297	2·97
Urea	20	11·9	241	2·03
$(NH_4)_2SO_4$	10	9·6	239	2·49
$(NH_4)_2SO_4$	20	9·9	228	2·31

has any obvious effect on the growth of the organism. Fig. 3 shows that the addition of the other stimulatory amino acids DL-serine and L-tyrosine did not enhance the effect due to L-threonine alone and that urea and $(NH_4)_2SO_4$ were also inactive. The addition of the purine adenine, however, enhanced the L-threonine effect and produced more riboflavin than could be expected from a simple summation of the increases obtained when these two substances were tested separately.

Similar experiments carried out using the basal medium containing 4 mg. adenine N/100 ml. and to which other nitrogen sources were added, again showed (Fig. 4) the extra stimulation in the presence of L-threonine and demonstrated that DL-serine was equally effective. Some stimulation was also obtained with urea and to a slight extent with L-tyrosine, whilst $(NH_4)_2SO_4$ was completely inactive.

It was thought that the failure of 5:6-dimethylbenziminazole and 1:2-dimethyl-4-amino-5-(D-1′-ribitylamino)benzene to stimulate riboflavin synthesis might be due to the absence of the appropriate coupling compound (e.g. alloxan). Consequently, they were tested in the presence of small non-inhibitory amounts of alloxan (4 mg. N/100 ml.) and adenine (4 mg. N/100 ml.) and DL-threonine (2 mg. N/100 ml.). In the first case there was no stimulation of riboflavin synthesis and in the latter there was no potentiation of the adenine and threonine effects.

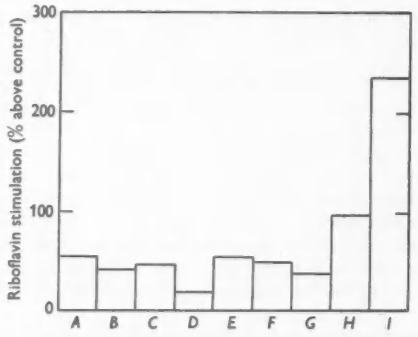


Fig. 3. The combined effect of L-threonine and one other N source on riboflavin synthesis. Results are expressed as % increase over production on the basal medium plus 0·005 % (w/v) peptone N. Cultural conditions as described in Fig. 1. *A*, L-threonine alone (1 mg. N/100 ml.); *B*, DL-serine alone (10 mg. N/100 ml.); *C*, *A* + *B*; *D*, L-tyrosine alone (5 mg. N/100 ml.); *E*, *A* + *D*; *F*, *A* + urea* (5 mg. N/100 ml.); *G*, *A* + $(NH_4)_2SO_4$* (10 mg. N/100 ml.); *H*, adenine alone (4 mg. N/100 ml.); *I*, *A* + *H*.

* $(NH_4)_2SO_4$ alone is without stimulatory action, whilst urea alone has a slight effect (Table 10).

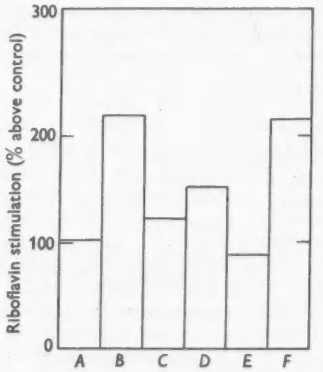


Fig. 4. The combined effect of the addition of adenine and one other N source on riboflavin synthesis. Results expressed as described in Fig. 3 and experimental conditions as described in Fig. 1. *A*, adenine alone (8 mg. N/100 ml.); *B*, *A* + L-threonine* (1 mg. N/100 ml.); *C*, *A* + L-tyrosine* (5 mg. N/100 ml.); *D*, *A* + urea* (5 mg. N/100 ml.); *E*, *A* + $(NH_4)_2SO_4$* (10 mg. N/100 ml.); *F*, *A* + DL-serine* (10 mg. N/100 ml.).

* For control values of the additional N source values see Fig 3 and Table 13.

Table 14. *The relative amounts of riboflavin in mycelium and medium*

Amounts produced in 15 ml. medium in 50 ml. conical flasks. Incubation time, 5 days; temp. 28°.

Medium	Riboflavin: In mycelium (μg.)	Riboflavin: In medium (μg.)	Riboflavin: Total (μg.)	% of total in mycelium
*A** (basal medium + 5 mg. peptone N/100 ml.)	131	181	312	41·7
*B** (basal medium + 5 mg. peptone N/100 ml.)	100	165	265	37·8
A + DL-threonine (2 mg. N/100 ml.)	168	263	431	39·0
A + adenine (4 mg. N/100 ml.)	153	297	450	34·0
B + glycine (20 mg. N/100 ml.)	59	115	174	34·0
B + 5:6-dimethylbenziminazole	78	119	197	39·6
B + 1:2-dimethyl-5-(D-1′-ribitylamino)benzene	82	188	270	30·4
B + 1:2-dimethyl-4-amino-5-(D-1′-ribitylamino)benzene	87	137	224	26·9

* *A* and *B* are the same medium but the two series of experiments were carried out at different times.

Riboflavin in the mycelium

According to Deseive (1947) the mycelial riboflavin (which is mainly in the form of flavin-adenine dinucleotide (Forrest, private communication)) in *E. ashbyii* is negligible compared with that in the medium. On checking this under our conditions this was found not to be so. Table 14 shows that about 30–40 % of the total riboflavin produced is in the mycelium. The possibility that the increased riboflavin observed in the medium in the presence of threonine, serine and purines might be due to the liberation of mycelial riboflavin into the medium is ruled out by the demonstration (Table 14) that in the presence of these stimulators the mycelial riboflavin is also increased, but not to the same extent as is the riboflavin in the medium. The results in Table 14 also show that the failure of glycine (which might be expected to be active because of its possible conversion into serine), 5:6-dimethylbenziminazole and 1:2-dimethyl-4-amino-5-(D-1′-ribitylamino)benzene to stimulate riboflavin synthesis is not due to the failure to release riboflavin from the mycelium into the medium.

DISCUSSION

Comparison with earlier work on amino acid utilization

It is somewhat difficult to compare the results of the present amino acid experiments with those of other investigators (Schopfer & Guilloud, 1945*b*; Chin, 1947; Deseive, 1947; Hickey, 1953) because the experimental conditions were so different and, apart from Hickey, none of these investigators examined either serine or threonine. In Hickey's experiments they were examined in culture media in which they were the sole source of nitrogen, and, as might be expected from the present work, they did not support growth of the organism.

In agreement with Chin (1947) and Hickey (1953) it has been found that asparagine, aspartate and glutamate were good nitrogen sources for both growth and riboflavin synthesis, and it is difficult to explain the results of Schopfer & Guilloud (1945*b*) which indicated that no growth or riboflavin was produced on aspartate or glutamate.

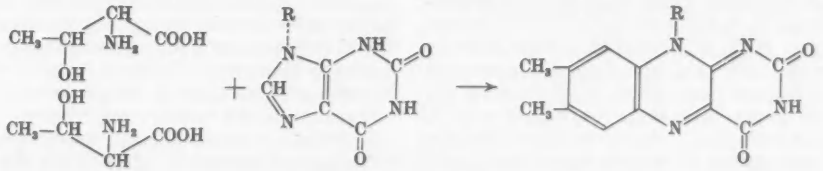
The present work confirms the observations of Deseive (1947) that L-cysteine inhibits considerably growth and flavinogenesis.

It is interesting that in the present experiments glycine had little if any effect on growth of *E. ashbyii* and these results tend to confirm those of Dulaney & Grutter (1950), who concluded that glycine was not utilized by this organism. Schopfer (1944), on the other hand, found that glycine stimulated growth in the presence of some samples of peptone and not in others. Most of the amino acids fall into the same category as glycine, but it ought to be emphasized that it is not necessarily true that they are not metabolized by *E. ashbyii*. If, for example, growth alone had been the criterion, then L-threonine would have been considered inert.

Role of amino acids and purines in biosynthesis of riboflavin

The most likely explanation of the stimulation of riboflavin synthesis in *E. ashbyii* by certain amino acids and purines is that they are sources of specific building units which, under the prescribed experimental conditions, are limiting factors. It is possible, however, that the effect might be indirect and, although this appears unlikely, it remains for isotope studies, at present in progress, to decide this unequivocally. There is, however, some evidence that in the closely related organism *A. gossypii*, the biosynthesis of riboflavin follows a very similar course to that of the purines. Recent work by Plaut (1953*a*) with this organism has shown that ^{14}C-labelled formate and CO_2 are incorporated almost entirely into C_2 and C_4 of riboflavin respectively. This agrees with the present views on the incorporation of these units into purines (see, for example, Brown, 1953). The position of glycine which is specifically incorporated into purines but which does not under our conditions stimulate riboflavin production appears to be somewhat anomalous. There is, however, no reason to believe that because it does not stimulate riboflavin synthesis it cannot be incorporated into the molecule, and very recently Plaut (1953*b*) has shown that incorporation can take place.

Assuming then the incorporation of the stimulatory amino acids and purines into riboflavin, many interesting possibilities arise. The fact that the amino acid stimulations are not cumulative suggests that they probably provide the same building unit. As the effect of amino acid plus purine is cumulative, the biosynthesis could be envisaged, using threonine and xanthine as examples, formally as follows:



On this scheme the amino acids function only by providing a carbon source, which is incorporated into the aromatic moiety of riboflavin. It is obvious that threonine and serine must provide the same or closely related units. It is not yet known whether the slight activity of tyrosine is possibly due to a limited ability of the fungus to split off serine from the molecule.

The observation that the addition of purine to a medium containing optimum amounts of L-threonine produces an effect greater than the sum of the two separate effects suggests that stimulation by L-threonine alone ceases at a low concentration because purines or their immediate precursors become the limiting factors. Addition of a purine results in the utilization of the remainder of the L-threonine and also in the stimulation of flavin biosynthesis due to the utilization of the purine itself; this is possible, for the conversion of purine into riboflavin is, even at low concentrations, only of the order of 20–30 %.

The fact that stimulation by purines alone does not stop at a comparatively low concentration but continues to increase even up to the highest concentration (a saturated solution) examined, would suggest that in the basal medium there is no shortage of 'aromatic precursors' such as may be provided by threonine. It could, however, also be ascribed to the fact that some of the purine is catabolized thereby yielding 'aromatic precursors'. It is, for example, possible from purely structural considerations, to envisage the release of serine.

A number of questions arise when the role of purines in flavinogenesis is further considered. They can only be solved by further work but may be briefly enumerated here: (*a*) although it is apparent that adenine can be deaminated, does this occur before or after incorporation into the riboflavin molecule? (*b*) is the ribose of adenosine removed or reduced to ribitol before incorporation of the purine? and (*c*) if 6:7-dimethyl*iso*alloxazine is first formed, is ribose attached and then reduced or is ribitol itself attached?

Role of o*-phenylenediamine derivatives and pyrimidines in riboflavin biosynthesis*

It is evident from this work that pyrimidines are not specifically incorporated into riboflavin, even in the presence of urea which, theoretically, could supply the unit for converting a pyrimidine into a purine. (The reason for the slight sporadic stimulation of urea alone is not yet obvious.) It has been found that in high concentration uracil actually inhibits riboflavin synthesis. This is in agreement with the original observations of Maclaren (1952), but we could not obtain as great an inhibition (50 % compared with 83 %). As it was found that the stimulatory power of adenine was reduced only slightly, in the presence of a considerable excess of uracil, Maclaren's suggestion that uracil may act by competitively inhibiting the incorporation of purines into the riboflavin molecule may not be correct.

The failure of 1:2-dimethyl-4-amino-5-(D-1'-ribitylamino)benzene (I) to stimulate the synthesis of riboflavin alone or in the presence of non-toxic amounts of alloxan strongly suggests that a biosynthetic pathway similar to the chemical synthesis of Banerjee, Dittmer & du Vigneaud (1945) involving the condensation of (I) with alloxan, is not the major pathway in this organism, if it occurs at all. Smith & Emmart (1949), however, did observe a slight stimulation of riboflavin synthesis in *Mycobacterium tuberculosis* with both (I) and alloxan.

The absence of stimulation of riboflavin synthesis by 5:6-dimethylbenziminazole (there is actually a slight inhibition) and (I) also makes it very unlikely that in *E. ashbyii*, at least, the pathway for riboflavin biosynthesis is similar to that for the cobalamins in other micro-organisms, e.g. *Escherichia coli*, where both 4:5-dimethyl-*o*-phenylenediamine and riboflavin itself stimulate the biosynthesis of cyanocobalamin in a vitamin B_{12}-requiring mutant (Ford & Holdsworth, 1954).

General aspects of amino acid metabolism

From the general point of view of amino acid metabolism, it is extremely interesting that serine is active in promoting flavinogenesis and glycine is not. Although glycine has been demonstrated to be the precursor of serine in many animal tissues and in some micro-organisms, e.g. *Torulopsis utilis* (Ehrensvärd, Sperber, Saluste, Reio & Stjernholm, 1947), it seems that *E. ashbyii* cannot bring about this conversion. From a similar point of view the activity of L-tyrosine and the inactivity of L-phenylalanine suggests that the latter cannot be converted into the former in *E. ashbyii*. This would appear to fit in with recent work on *Esch. coli* (Simmonds, Tatum & Fruton, 1947; Davis, 1950) which suggests that there are independent routes of synthesis of these two amino acids and that phenylalanine is not converted into tyrosine.

SUMMARY

1. *Eremothecium ashbyii* grown on a basal glucose–salts–vitamins medium supplemented with 0·24 % (w/v) peptone is fully grown after 5 days but continues to produce riboflavin up to 7–8 days. Maximal concentration of riboflavin is obtained over the range 0·16–0·40 % (w/v) peptone.

2. Using the basal medium supplemented with 0·005 % (w/v) peptone N, addition of L-threonine,

L-serine or L-tyrosine specifically stimulated riboflavin synthesis; L-tyrosine was the least effective. D-Threonine was inactive. L-Glutamate, L-aspartate and L-asparagine stimulated both growth and flavinogenesis, whilst L-cysteine inhibited both. Other amino acids tested were without obvious effect on riboflavin synthesis, but some inhibited growth slightly.

3. Under the same conditions the purines xanthine and adenine, and adenosine, stimulated flavinogenesis whilst most pyrimidines and their derivatives were without effect, although alloxan inhibited both growth and riboflavin synthesis. At very high concentrations, uracil inhibited riboflavin synthesis, although it did not inhibit the adenine-stimulated production of riboflavin.

4. Neither 5:6-dimethylbenziminazole nor 1:2-dimethyl-4-amino-5-(D-1′-ribitylamino) benzene stimulated riboflavin production under the same conditions.

5. Of the other nitrogen-containing compounds tested, urea in low concentrations occasionally stimulated and $(NH_4)_2SO_4$ was without effect. D-Glucosamine was a powerful inhibitor of both growth and riboflavin.

6. The effects of the stimulatory amino acids are not cumulative, whilst those of a purine and an amino acid are.

7. The significance of these results in relation to the problem of the biosynthesis of riboflavin is discussed.

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On the Significance of γ-Glutamyl Transpeptidation in Peptide Biosynthesis

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Transpeptidation reactions have been receiving increasing emphasis in connexion with the syntheses of peptide and amide bonds and hence of proteins (Hanes, Hird & Isherwood, 1950, 1952; Hanes, Connell & Dixon, 1953; Waelsch, 1953; Fruton, Johnston & Fried, 1951; Fruton, 1950). Particular interest attaches to the γ-glutamyl transpeptidation reaction of Hanes *et al.* (1950, 1952) because the hypothesis has been advanced that glutamine and glutathione serve to direct the energy of the energy-rich bonds of adenosine triphosphate into the formation of peptide bonds by means of the transpeptidation and transamidation reactions (Waelsch, 1953). The hypothesis further assumes that the γ-glutamyl peptides formed by these reactions would either rearrange to α-peptides or by further exchanges

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between themselves or other amino acids form α-peptide linkages. The experimental basis for the theory is that glutathione incubated with a substrate amino acid in the presence of a dialysed extract from fresh sheep kidney or certain other tissues yielded the corresponding γ-glutamyl peptides, of which γ-glutamylphenylalanine and γ-glutamylglycine have been identified (Hanes *et al.* 1952).

The object of the present investigation was to test the γ-glutamyl transpeptidation hypothesis in several tissue systems known to be capable of incorporating amino acids into the cellular protein material. The work involved two approaches, one to study the effect of the addition of γ-glutamyl peptides to tissues undergoing active amino acid incorporation, the other an attempt to demonstrate the natural occurrence of γ-glutamyl peptides formed from ^{14}C-labelled subtrates. It will be seen that the results of this study cannot be interpreted as being favourable to the γ-glutamyl transpeptidation hypothesis. A preliminary report of the results has been published (Hendler & Greenberg, 1952).

METHODS

^{14}C-compounds. The isotopic compounds were [1:2-^{14}C]-glycine, DL-[2-^{14}C]valine, DL-[4:4′-^{14}C]valine hydrochloride, DL-[3-^{14}C]phenylalanine hydrochloride (obtained from Tracerlab Inc., Boston, Massachusetts), and γ-glutamyl-[1:2-^{14}C]glycine, synthesized as described below.

Synthesis of γ-glutamylglycine

The steps of the reactions utilized are: glutamic acid→γ-ethyl L-glutamate→γ-ethyl benzyloxycarbonyl-L-glutamate (γ-ethyl BOC-L-glutamate)→BOC-γ-L-glutamylhydrazide→BOC-γ-L-glutamylazide→BOC-γ-L-glutamylglycine →γ-L-glutamylglycine.

γ-Ethyl L-glutamate (Hanby, Waley & Watson, 1950), γ-ethyl BOC-L-glutamate (Hegedus, 1948) and BOC-γ-L-glutamylhydrazide (LeQuesne & Young, 1950) were prepared by the standard procedures described in the references cited. The γ-ethyl BOC-L-glutamate, after being taken up in ether, was washed with water and dried *in vacuo* over P_2O_5. This material, without further purification, was employed to prepare the BOC-γ-L-glutamylhydrazide, which, upon recrystallization from water, yielded white needles (m.p. 179–180°).

The synthesis was carried out on a micro scale. To secure a maximum yield based on glycine it was found desirable to increase the molar ratio of BOC-γ-L-glutamylhydrazide to glycine over that used by LeQuesne & Young. On a 0·1 molar scale, by increasing the molar ratio of hydrazide to glycine from 0·64 to 1·0, the yield of recrystallized BOC-γ-L-glutamylglycine was increased from 20 to 61%, based on glycine. By this step it was possible to obtain 110 mg. of recrystallized BOC-γ-L-glutamylglycine, which yielded 26·2 mg. of twice-reprecipitated γ-L-glutamylglycine after catalytic hydrogenation. The yield of pure γ-L-glutamylglycine on the micro-scale synthesis was 24% (based on glycine) compared with the overall yield obtained by LeQuesne & Young of the same product (not reprecipitated) of 16%.

Synthesis procedure. BOC-γ-L-glutamylhydrazide (157 mg.) was dissolved in 2·14 ml. water containing 0·2 ml. of 12N-HCl in a 10 ml. separating funnel having a side arm with a ground glass joint. After addition of ethyl acetate (1·35 ml.), a miniature vacuum-driven stirring motor with a 1 mm. bent glass rod stirrer was placed above the solution. The separating funnel was almost completely immersed in a beaker of ice-water in the cold room. With vigorous stirring a solution of $NaNO_2$ (61·8 mg.) in water (0·18 ml.) was added drop by drop through the side arm over 5 min. Rapid stirring was continued for a further 10 min. The ethyl acetate layer was separated and washed twice with cold water (about 0·2 ml. each). It was then added slowly with stirring from the 10 ml. separating funnel into the side arm of a 5 ml. separating funnel containing [1:2-^{14}C]glycine (40 mg., 2·5 μc/mg.) and $KHCO_3$ (188 mg.) dissolved in water (1 ml.), which was cooled in ice-water as above. The 10 ml. funnel was rinsed with 0·2 ml. ethyl acetate which was added to the glycine-containing funnel. Stirring was continued for 15 min. in the cold and the separating funnel left for 2·5 hr. at room temp. and then for 10–15 min. in warm water (about 40°). (Sometimes about 0·3 ml. of fresh ethyl acetate was added to replace loss of ethyl acetate by evaporation at room temperature and at 40°.) The aqueous layer was acidified with 12N-HCl, and extracted 4 times with 1 ml. portions of ethyl acetate. The combined extracts were washed with water (about 0·3 ml.) and placed in a 10 ml. beaker. Concentration was effected by placing the beaker covered with Parafilm (Polythene) in a vacuum-filtering device (Fisher Filtrator) fitted with a long capillary (containing glass wool) through a rubber stopper at the top. The capillary tip was introduced through the Parafilm just above the liquid, and upon evacuation of the Filtrator a fine jet of air was played upon the solution. This apparatus, illuminated by i.r. lamps, gave a very effective means for easy concentration with minimum loss of material. The remaining oil was washed with ether and left in a vacuum desiccator over P_2O_5 overnight (yield 141·6 mg.). This was recrystallized by dissolving it in 7 drops of boiling water and allowing it to cool slowly while its container (a 5 ml. beaker) was immersed in a larger beaker of hot water. The ppt. was filtered and washed with copious quantities of cold water (yield 110 mg.). The BOC-γ-L-glutamylglycine (110 mg.) was dissolved in 0·5 ml. of methanol containing 0·02 ml. glacial acetic acid and 2 drops of water in a micro-bubbling apparatus, palladous oxide (12·5 mg.) was added and, with the tip of a steel pin encased in a glass capillary tube (1 × 5 mm.) to serve as a magnetic stirrer, a stream of hydrogen was passed through. After 37 min., methanol (0·5 ml.), glacial acetic acid (0·02 ml.) and water (2 drops) were added to the reaction flask to replace that lost by evaporation. After about 1 hr. no further CO_2 was evolved, showing that the reaction was complete. The weight of $BaCO_3$ collected from the effluent gas was 55 mg., indicating a maximum possible yield of 57 mg. of γ-glutamylglycine. The reaction tube was centrifuged, the supernatant fluid sucked into a drawn out capillary tube fitted with a rubber bulb and filtered through Whatman no. 1 paper into a small beaker. The remaining material in the reaction flask was washed with (0·5 ml.) water and this also was filtered into the beaker. The contents of the beaker were evaporated to dryness by placing the open end of a sealed capillary tube in the liquid and putting the beaker in a desiccator over P_2O_5 under

reduced pressure; yield of crude γ-glutamylglycine, 52·4 mg. The material was twice dissolved in 15 drops of water and precipitated by adding 68 drops of ethanol. It was filtered through Whatman no. 50 filter paper supported on an inverted filter stick. Yield 26·2 mg. (24%). (Found: C, 41·3; H, 6·0; Calc. for $C_7H_{12}O_5N_2$: C, 41·2; H, 5·9; N, 13·7%. Found for a sample of unlabelled peptide prepared by the identical procedure: C, 41·3; H, 5·9; N, 13·6%.)

Preparation of samples and counting of radioactivity

The isolation and treatment of protein and other materials for counting of radioactivity, treatment with mercaptoethanol, and plating have been described in previous publications (Peterson & Greenberg, 1952; Lien & Greenberg, 1952). Briefly this consisted of precipitating the proteins with trichloroacetic acid (TCA) (final concentration about 7%, w/v) after incubation, washing with hot and room-temperature TCA solutions, and dehydrating and preparing the proteins for plating with washes of ethanol, ethanol–ether, ether, and ether–light petroleum–acetone. To remove disulphide-bound material, when desired, the protein was suspended for 20–30 hr. under N_2 at room temperature in a mercaptoethanol solution. It was then reprecipitated with TCA, washed and plated as above and recounted. Protein was plated on aluminium disks of large diameter (1·75 in.) and the fraction from Dowex or Amberlite columns were received and evaporated to dryness in polyethylene cups (Lien & Greenberg, 1952) and counted in windowless gas-flow counters. In the case of paper chromatograms, radioactive spots were detected by means of a scanning windowless gas-flow counter tube held over each 0·75 in. of the path of the spots for 30 sec., using a T-square to guide the counter in its path.

Enzyme preparations and incubation procedures

Cell particles–supernatant fluid (PS preparation). In a typical experiment, the washed liver (approx. 9 g.) of a 200–230 g. rat was homogenized cold in an equal weight of buffer (0·12 M-KCl + 0·04 M-$KHCO_3$ equilibrated with 95% O_2–5% CO_2). The particulate fraction was isolated by differential centrifuging (Peterson & Greenberg, 1952) and suspended in an equal volume of the liver supernatant fluid obtained by centrifuging part of the above homogenate for 30 min. at 4000 rev./min. Portions (0·3 ml.) of this preparation were added to 12 ml. centrifuge tubes containing the radioactive amino acid and the other desired constituents, which had been evaporated to dryness in the tubes in vacuum at 40°, in 0·03 ml. of water (final vol. 0·33 ml.). Incubation was performed at 37° in a rotating circular rack immersed in a constant temperature bath.

Cell particles–synthetic medium (PM preparation). The same particulate fraction as above was suspended in a synthetic incubation medium, yielding final concentrations of 6 mM citrate, 10 mM Mg^{2+} and 3 mM ATP. In addition, the medium contained a mixture of amino acids shown to have an activating effect (Peterson & Greenberg, 1952), namely, 1·6 mM methionine, 2 mM aspartate, 3 mM glutamate, 1·2 mM arginine, 1 mM proline and 2·2 mM alanine.

Gardner mouse lymphosarcoma (6C3H-ED). The mouse tumours were pooled and forced through a 60-mesh monel metal screen (Farber, Kit & Greenberg, 1951) to yield a suspension of essentially free cells.

Mouse spleen. Suspensions of cells were prepared as described for the lymphosarcoma.

Mouse spleen homogenate. The cell suspension obtained as above was homogenized for 5 min. at 0° with 2 vol. of buffer in a tight-fitting all-glass homogenizer. Microscopic examination, after staining in methylene blue in 1% (v/v) acetic acid, showed that about 50% of the cells were ruptured.

Kidney homogenate. One g. of rat kidney was homogenized with 6 ml. of the buffer described by Hunter (1949), containing also glucose (11·4 mg.) and $KHCO_3$ (7·2 mg.).

Hanes et al. (1952) *transpeptidase preparation.* This was prepared as described by these authors from a sheep kidney obtained in the laboratory within 25 min. of the death of the animal. Various modifications of the above enzyme preparation were tried with rat kidney. The manner of dialysis was varied; the extract was employed in different dilutions from full strength to 1/4 strength, the incubations were carried out both at 32 and 37°, and the buffer concentration was varied between 0·017 and 0·05 M.

The incubation volumes of the Peterson & Greenberg systems were 0·33 ml., of all others 1·0 ml. All incubations were carried out for 60 min. at 37° unless otherwise noted. When mM concentrations of substrates are mentioned in the text or tables they represent final concentrations.

Chromatographic procedures

Paper chromatography (Whatman no. 4 and/or no. 1 paper) was performed with the solvent systems: 80% (v/v) propanol–water (Hanes *et al.* 1950), butanol–acetic acid–water (100:22·5:50, v/v), and phenol–water (100 g. of reagent grade phenol, distilled over zinc at atmospheric pressure, dissolved in 38 ml. water).

Dowex column chromatography. The columns (55 × 1 cm.) were prepared by standard methods (Stein & Moore, 1950; Lien & Greenberg, 1952), using Dowex-50 (Micro-chemical Specialities Co., Berkeley, California) in the acid form and were eluted with 1·5 N-HCl alone, except in the case of phenylalanine, when this was followed by 6 N-HCl. The samples were applied in about 0·5 ml. vol. and represented the supernatant fluid from the different tissue preparations after addition of TCA to about 7% (w/v) concentration.

Amberlite ion-exchange. Amberlite IR–4B (Resinous Products Corp., Philadelphia, Pa.) in a column 3 × 1 cm. was generated into the NH_2 form by approximately 100 ml. of N-NaOH. Extensive washing with water brought the effluent pH to that of distilled water. 3 ml. of clear supernatant fluid from an incubation were passed through Amberlite IRC–50 in the acid form (column 2·5 × 1·3 cm.) and the first 10 ml. of effluent was applied to IR–4B. Water was passed through the basic resin so that 30 ml. were collected in a 50 ml. volumetric flask which had 20 ml. of water carefully pipetted into it. This fraction contained all materials not adsorbed to the column. A second fraction of 4 ml. was then taken to assure that the radioactivity was at the base line, after which elution was accomplished with N-HCl. The third fraction consisted of 20 ml. of this eluate collected in a 25 ml. volumetric flask containing 5 ml. water. 1 ml. portions from each of the three fractions (further diluted when necessary) were pipetted into polyethylene planchets in triplicate and counted. The HCl eluate from the IR–4B resin is referred to as the acidic peak.

RESULTS AND DISCUSSION

Amino acid incorporation

The incorporation and Dowex chromatographic experiments were performed with glycine, valine, and phenylalanine. These amino acids were chosen because Hanes *et al.* (1950, 1952) reported their corresponding glutamyl peptides, and because they would be expected to be involved in any general scheme for amino acid incorporation. The most thorough study was made with glycine for which both the radioactive and the unlabelled γ-glutamyl peptide were available. In every case the label was more efficiently incorporated into the cellular proteins when the incubation was carried out with free glycine than with an equimolar quantity of γ-glutamylglycine (Table 1).

In the PS system, the glycine of the γ-glutamyl peptide was utilized 73, 63 and 87 % as efficiently as free glycine. To eliminate the possibility that the amount of γ-glutamyl peptide used was in excess of an optimum effective concentration, two lower concentrations were tried. The incorporation in these experiments was correspondingly lower.

Mercaptoethanol treatment, which has been shown by Peterson & Greenberg (1952) to remove an appreciable amount of radioactivity from the proteins of the PS system after incubation with glycine, did not alter the results. As much or more activity was removed by this treatment from the proteins after incubation with γ-glutamylglycine, than with glycine. The proteins of whole-cell suspensions were resistant to this treatment.

The spleen whole-cell suspension used the peptide glycine only 24 and 18 % as efficiently as free glycine. Since the relative amount of glycine incorporated into TCA-insoluble protein of the spleen system was only about one-third that of the cell-free liver system, the question of cell-membrane permeability arose. Tests with a spleen homogenate showed the same relative incorporation of the peptide glycine with respect to the free glycine as for the whole-cell suspension (although the actual uptake was much less).

A cell suspension of the Gardner lymphosarcoma (6C3H-ED) from mice used the peptide glycine approximately as effectively (13 % compared with glycine) as its analogous tissue (the spleen).

Rat kidney homogenate showed very slight fixation of [^{14}C]glycine into the insoluble protein. Uptake was not improved by supplying the glycine peptide.

The addition of glutamic acid, glutamine, glutathione, or unlabelled γ-glutamylglycine had no significant effect on the uptake of glycine, except in the spleen and Gardener lymphosarcoma cell suspensions, in which stimulation by these compounds is indicated (Table 2).

The uptake of valine in the PS system was not significantly influenced by addition of the same glutamyl compounds listed above (Table 2). Since the PS system might already be saturated with γ-glutamyl compounds (this is extremely unlikely from the results to be described in the section on chromatography with Dowex) a PM system employing a synthetic medium was tried, and this too showed no activation. In another experiment, ATP and citrate were omitted from the synthetic medium since the tissue might have been able to synthesize adequate amounts of γ-glutamyl peptide with this energy supply. Uptake dropped and no significant activation was shown by addition of unlabelled γ-glutamylglycine.

The kidney homogenate system definitely was not activated towards the incorporation of phenylalanine by addition of the glutamic acid-containing compounds; in fact, an inhibition by these compounds was observed.

Table 1. *Incorporation of radioactive glycine into the proteins of suspensions of various tissues*

Tissue suspension incubated with [1:2-^{14}C]glycine or γ-glutamyl-[1:2-^{14}C]glycine (GG) for 60 min. at 37°. Proteins were precipitated and washed as described in the text. The figures in columns 3–5 are the mean percentage ± the mean deviations of the corresponding glycine values arbitrarily taken as 100. Unless otherwise indicated, the final concentration of the labelled compounds was 1·2 mM.

	Glycine			
Enzyme system	(Counts/min./mg. protein)	(%)	GG (% of glycine)	GG (% of glycine)
PS	26	100±11	73±2	42±9 (0·4 mM)
PS	33	100±14	63±1	16±1 (0·24 mM)
PS	27	100±3	87±2	—
Spleen whole cells	200	100±10	24±1	8±0 (0·24 mM)
Spleen whole cells	175	100±10	18±0	—
Spleen homogenate	53	100±15	18±5	—
Lymphosarcoma	32	100±5	13±4	—
Kidney homogenate	3·2	100±19	67±34	—

Table 2. *Incorporation of radioactive amino acids in presence and absence of glutamyl compounds*

Incubations were for 60 min. at 37°. Proteins were precipitated and washed as described in the text.

Enzyme system	Labelled substrate†	None		Cosubstrates*							
				Glutamic acid		Glutamine		Glutathione		γ-Glutamylglycine	
		(Counts/min./mg. protein)	(%)	(%)	(R‡)	(%)	(R‡)	(%)	(R‡)	(%)	(R‡)
PS	Glycine	26	100±11	78±2	1	87§	1	—	—	—	—
PS	Glycine	33	100±14	100±14	1	82§	1	92±15	0·1	97±2	0·1
PS	Valine‖	31	100±5	100±4	0·1	97±1	0·1	104±3	0·1	—	—
PS	Valine‖	26	100±9	111±5	0·1	108±8	0·1	107±11	0·01	—	—
PS	Valine¶	18	100±3	96±6	0·33	—	—	100±1	0·06	88±8	0·06
PM	Valine¶	93	100±4	90±4	0·33	—	—	—	—	95±6	—
PM (citrate and ATP absent)	Valine¶	51	55±1	—	—	—	—	—	—	37±5	0·03
PM	Valine¶	31	100±4	93±2	0·33	—	—	—	—	131±6	0·03
PM (citrate and ATP absent)	Valine¶	22	63±6	—	—	—	—	—	—	77±0	0·03
Spleen whole cells	Glycine	200	100±10	125±2	1	123±1	1	—	—	127±2	0·1
Spleen homogenate	Glycine	53	100±15	95±5	1	117	1	—	—	—	—
Lymphosarcoma	Glycine	32	100±5	95§	1	140±29	1	—	—	147±4	0·1
Kidney homogenate	Glycine	3·2	100±19	67±13	1	—	—	—	—	78±28	0·1
Kidney homogenate	Phenylalanine	29	100±2	87±1	0·44	84±6	0·44	104±0	0·44	77±11	0·44
Kidney homogenate	Phenylalanine	38	100±6	—	—	—	—	107±3	0·44	—	—
Kidney homogenate	Phenylalanine	46	100±9	39±1	0·13	21±3	0·13	56±1 59±3	0·13 0·26	42±1 —	0·05 —
Kidney homogenate	Phenylalanine	12	100±14	95±7	0·13	41±1	0·13	79±3	0·13	91±18	0·05
Kidney homogenate	Phenylalanine	103	100±9	44±4	0·13	—	—	—	—	43±1 36±1	0·13 0·26

* Uptake of the free amino acid in absence of cosubstrates but in otherwise complete systems (col. 3) is arbitrarily taken as 100 (col. 4). Figures (mean percentage ±deviation) in cols. 5, 7, 9 and 11 are relative to this arbitrary standard. The figures (col. 4) referring to the PM system without citrate or ATP are also relative to this standard.

† Final concentrations of labelled amino acids were: glycine, 1·2 mM; valine, 4 mM; phenylalanine, 0·9 mM in upper three and 1·5 mM in last two experiments.

‡ $R = \frac{\text{molar concentration of cosubstrate}}{\text{molar concentration of substrate}}$.

§ Single determination.

‖ Valine labelled in α-carbon.

¶ Valine labelled in methyl carbons.

Chromatography with Dowex-50

These fractionations were undertaken to determine the nature of the products formed from the various radioactive substrates employed in the experiments. A summary of representative results obtained is given in Table 3. The fractionation of the supernatant fluid from incubations with γ-glutamylglycine for 0·5, 20 and 60 min. on a Dowex-50 column revealed that glycine and serine appeared quite readily at the expense of the peptide in the PS system and more readily in the rat kidney homogenate system. Experiments with supernatant fluids from glycine incubations showed that serine is readily formed from glycine, and that no γ-glutamylglycine appeared in incubations carried out for 20 or 60 min. or for 20 min. when unlabelled γ-glutamylglycine was added as a 'trap' or as γ-glutamyl donor source. Of interest in the comparison of chromatograms from the spleen and kidney is the unknown peak which appeared when γ-glutamylglycine was incubated, but not when free glycine was used. This may well be α-keto-γ-glutarylglycine (γ-carboxy-γ-oxobutyrylglycine). The position of the spot on the autoradiograph is in keeping with a compound more acidic than γ-glutamylglycine. The increase with time of this peak in the spleen experiment, and the decrease with time in the kidney experiment would parallel the respective abilities of the two systems to cleave the γ-glutamyl bond.

The stability of the γ-glutamylglycine in mouse spleen is in striking contrast to its lability in the other two systems. Evidence that the γ-glutamyl peptide bond is stable to hydrolysis under the acidic conditions of chromatography on Dowex-50 can be seen in the experiment with spleen (Table 3). When the TCA supernatant from a 0·5 min. incubation was chromatographed on Dowex-50, over 99% of the radioactivity was in the discrete peak for γ-glutamylglycine. No activity was detected in glycine. Correspondingly, after incubation for 20 and 60 min., column development of the supernatant showed 97 and 93% of the radioactivity, respectively, in γ-glutamylglycine, whereas 2 and 5%, respectively, were in glycine. Radioactive γ-glutamylglycine also was chromatographed on Dowex-50 with no previous incubation, and no detectable counts appeared as glycine. The apparent ease of hydrolysis of the peptide in the liver and kidney is contrary to the proposed function of the γ-glutamyl link as a stable source of peptide bonds (by themselves or in adjacent α-peptide bonds, as in glutathione). That the disappearance of peptide and appearance of labelled glycine represents hydrolysis and not transpeptidation is supported by the fact that added unlabelled γ-glutamylglycine in the same systems was inert as a participant in transpeptidation with free labelled glycine, valine or phenylalanine (Table 3).

It is worth noting that the relative efficiency of utilization of the peptide glycine runs parallel to the ability of the system to hydrolyse the peptide. This suggests that prior hydrolysis is necessary for incorporation of glycine introduced from the γ-glutamylglycine.

In incubations with valine in the PS system at 20 and 60 min., and at 20 min. in the presence of unlabelled γ-glutamylglycine, no peak appeared which could be assigned to γ-glutamylvaline. If the compound was formed it would have to be in an amount less than 0·1% of the added valine. Methyl-labelled valine showed a significant conversion into aspartic acid, glutamic acid, and glycine and/or alanine. In addition, a prominent forepeak (or peaks) occurred (Table 3). This would indicate that the label readily enters the tricarboxylic acid cycle and that transamination is active in this system. The forepeak is indicative of labelled tricarboxylic acid cycle intermediates or of non-volatile fatty acids.

The supernatant fluid from an incubation with phenylalanine in rat kidney homogenate at 20 and 60 min., and at 20 min. in the presence of unlabelled γ-glutamylglycine showed that all the activity was retained in phenylalanine and in a broad forepeak which diminished markedly from 10% at 20 min. to 2% at 60 min. No evidence was obtained of the formation of γ-glutamylphenylalanine (Table 3). It could not have been present in the forepeak because this peptide would be expected to be retained more strongly than γ-glutamylglycine by the column.

γ-Glutamyl compounds may need concentrations corresponding to only a few hundredths of a per cent of the added labelled amino acid for an effective role in transpeptidation. However, the experiments with added labelled γ-glutamylglycine in diminishing amounts are not in accord with this possibility. It is also important to point out that the rather substantial conversions obtained by Hanes *et al.* (1950, 1952) do not occur in these systems which are incorporating amino acids into proteins.

Amberlite ion-exchange

If a neutral amino acid forms an acidic metabolite, an acid-adsorbing resin should be applicable to assay the extent of conversion. γ-Glutamyl peptides would be expected to have a degree of acidic character similar to glutathione which has a lower isoelectric point than glutamic acid. Amberlite IR–4B was used by Cannan (1944) to remove glutamic and aspartic acids quantitatively from protein hydrolysates. Chromatography with this resin appeared to us to be applicable to screen for further study the several different tissues for the

Table 3. *Radioactive fractions obtained by Dowex-50 chromatography from protein-free incubation fluids*

After incubation, a TCA extract (vol. below 1 ml.) of the homogenized tissue was introduced in the column and developed with 1·5 N HCl.

Radioactive substrate	Enzyme system	Incubation time (min.)	Total radioactivity of fluid† (counts/30 sec.)	Percentage of total radioactivity*					
				γ-Glutamyl-glycine	Glycine	Serine	Alanine	Phenyl-alanine	Forepeak or unknown compounds
γ-Glutamyl-[1:2-^{14}C]glycine	Liver-PS	0·5	36 200	97	3	—	—	—	—
		20	41 250	13	73	13	0·6	—	—
		60	24 900	0·8	80	17	2·0	—	—
	Intact spleen cells	0·5	42 150	99	—	—	—	—	0·3‡
		20	47 000	97	2	—	—	—	1
		60	39 700	93	5	—	—	—	2
	Kidney	0·5	34 400	54	44	—	—	—	3
		20	46 150	—	98	2	—	—	0·3
		60	31 000	—	97	3	—	—	—
1:2-^{14}C]Glycine	Liver-PS	20	24 300	—	78	21	—	—	1§
		20‖	27 800	—	79	20	—	—	1
		60	21 800	—	71	23	—	—	6
	Intact spleen cells	20	38 800	—	99	1	—	—	—
		20‖	35 400	—	99	1	—	—	0·3
		60	35 650	—	98	2	—	—	—
	Kidney	20	25 500	—	87	13	—	—	—
	Homogenate	20‖	30 500	—	90	10	—	—	—
		60	31 500	—	89	11	—	—	—
DL-[3-^{14}C]Phenylalanine	Kidney	20	61 000	—	—	—	—	92	8‡
	Homogenate	20	65 100	—	—	—	—	90	10
		60	67 100	—	—	—	—	98	2

				Valine	Alanine or glycine	Serine	Glutamic acid	Aspartic acid	Forepeak or unknown compounds
DL-[4:4′-^{14}C]Valine	Liver-PS	20	259 000	93	—	—	—	5	2‡
		20*	259 800	93	0·3	0·8	1·4	2	2
		60	217 600	90	1	1·5	2·0	0·2	6

* Compounds named were identified by paper chromatography against known compounds; solvents employed, phenol–water and butanol–acetic acid (see text).
† Obtained from summation of radioactivity in all peaks.
‡ Radioactivity in this group present in unknown compound or compounds.
§ Radioactivity in this group present in forepeak.
‖ Unlabelled γ-glutamylglycine added.

system most abundant in γ-glutamyl peptides and thus, in all probability, most active in γ-glutamyl synthetic activity and utilization.

Control experiments showed that Amberlite IR–4B retained practically no valine (less than 0·1 %), but had a strong affinity for acidic substances, such as glutamic acid, propionic acid, glutathione and γ-glutamylglycine. This resin therefore appeared to be ideal for studying the order of magnitude of the formation of γ-glutamyl peptides.

The technique was applied to a study of several different enzyme systems involving different amino acids, different methods of preparing the cell extract, and the reduction of permeability barriers of intact cells. The acids retained should then be indicative of the upper levels of formation of γ-glutamylpeptides, glutamic and aspartic acids, and any other acidic products which would also be retained. The results indicated minute upper levels for the formation of acidic peptides (a few tenths of 1 %).

Unfortunately it was found later that the salts in the concentrations present in the buffers of the incubation media seriously interfered with the adsorptive ability of the resin. γ-Glutamylglycine, in the absence of buffer, was retained to the extent of 67 %. In the presence of buffer only 12 % was retained. The correction necessary was too great to attribute any quantitative significance to these experiments, but as an order of magnitude, it can probably be safely said that in all the incubation systems employed, the extent of conversion, if any, was less than 5 %.

Hanes, Hird & Isherwood systems

Attempts to reproduce the observations by Hanes *et al.* (1950, 1952) were conducted with radioactive amino acids on rat kidney and in one series of experiments on sheep kidney. All of the other details in the procedure as described by these authors were very carefully followed, and in addition many modifications were tried with rat tissue. Radioactive valine was used in all experiments and radioactive phenylalanine was included in the experiment with sheep kidney. In addition to the 50 mM concentrations employed by Hanes *et al.*, experiments were performed with one-tenth of these concentrations, which are undoubtedly closer to average physiological concentrations. Evidence of enzymic activity in these preparations was given by the appearance of cysteine and cysteinylglycine (identified by its R_F value) at the expense of glutathione. At the 5 mM concentration the glutathione spot completely disappeared and was replaced by the hydrolysis products. At the amino acid concentrations of Hanes *et al.*, the radioactivity introduced as valine or phenylalanine travelled only with the corresponding amino acid. There were a few sporadic cases, however, in which a slight peak of radioactivity appeared in an area that might be expected for γ-glutamylvaline. No ninhydrin colour was evident in these areas and the amount of radioactivity relative to valine was less than 1 %.

Likewise with phenylalanine in the sheep kidney preparation at the lower concentration (4 mM), but not at the concentration of Hanes *et al.* (50 mM), a small peak of radioactivity appeared in an area that might be expected for γ-glutamylphenylalanine. This too yielded no ninhydrin colour. All the other ninhydrin spots described by these authors were observed.

We have no explanation for the failure to demonstrate the enzymic formation of γ-glutamyl peptides, although we carefully followed the instructions of Hanes *et al.*, employed radioactive tracers which should have greatly increased the ease of detection, and varied the experimental conditions in a variety of ways. It would appear that the γ-glutamyl transpeptidase enzyme system is subject to loss of activity by rather subtle differences in the preparation procedure.

In this connexion it is worthy of note that the formation of γ-glutamylarginine by ox-kidney extracts from glutathione or γ-glutamylglycine and arginine, reported by Kinoshita & Ball (1953) required greatly different experimental conditions from those of Hanes *et al.* The optimum amino acid and glutathione concentrations were 5 mM rather than 50 mM, and the optimum pH 9·0 instead of 7·4. Hanes and co-workers, found arginine to be a poor γ-glutamyl peptide former in their experiments.

More important than the demonstration of the accumulation of γ-glutamyl peptides is the question of their significance in a general scheme of peptide biosynthesis and protein formation. The work reported in this paper is not favourable to a hypothesis involving such a role for γ-glutamyl compounds.

SUMMARY

1. In a study of the significance of the γ-glutamyl transpeptidation hypothesis for the biosynthesis of peptides in various tissue preparations, it was found that free glycine was always incorporated into protein more efficiently than glycine from γ-glutamylglycine and no favourable influence of the γ-glutamyl peptide was observed on the incorporation of radioactive glycine.

2. The incorporation of glycine, valine or phenylalanine into protein was not increased by the addition of glutamic acid or γ-glutamyl compounds.

3. Chromatographic fractionation on Dowex-50 of the supernatant fluids of tissue preparations incubated with γ-glutamyl glycine showed that this peptide is rapidly hydrolysed by liver and kidney

homogenates, but is remarkably stable in the spleen. The chromatogram showed no evidence of the formation of the corresponding radioactive γ-glutamyl peptides from incubations with radioactive glycine, valine or phenylalanine in the presence or absence of unlabelled γ-glutamylglycine.

4. The formation of acidic metabolites, including γ-glutamyl peptides, from neutral substances was studied using chromatography with Amberlite IR–4B resin. An upper level of 5 % was obtained as the possible extent of formation of γ-glutamyl peptides.

5. Attempts to confirm the formation of γ-glutamylvaline and γ-glutamylphenylalanine, employing radioactive amino acids, by the sheep kidney system of Hanes *et al.* (1950, 1952) and modifications of this system with rat kidney were unsuccessful.

6. A micro synthesis of γ-glutamyl-[1:2-^{14}C]-glycine is described.

[1:2-^{14}C]Glycine and DL-[2-^{14}C]valine were synthesized under the direction of Dr E. M. Gal of this Department, the DL-[4:4′-^{14}C]valine was prepared by the Bio-organic Group of the Radiation Laboratory of the University by R. Ostwald, P. T. Adams and B. Tolbert (unpublished method). We are indebted to Mr G. Ellis of this Department for construction of the vacuum-driven stirring motor and the scanning windowless gas-flow counter. The paper represents work condensed from a thesis submitted by R. W. H. to the Graduate division of the University of California for the degree of Ph.D., June 1952, and was aided by research grants from the cancer research funds of the University of California and the Hobson Fund of the School of Medicine.

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Techniques in Tissue Metabolism

3. STUDY OF TISSUE FRAGMENTS WITH LITTLE OR NO ADDED AQUEOUS PHASE, AND IN OILS

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In conducting metabolic experiments with fragments of tissues, a common procedure during the past 30 years has been to suspend the tissue in some 30–200 times its volume of aqueous fluid. The artificial nature of this situation can be appreciated by noting that the volume of blood in an animal tissue at any one time is usually between one-thirtieth and one-third of its total volume. Even consideration of the rate of blood flow *in vivo* does not always justify the high fluid/tissue ratios which have been employed *in vitro*. Thus blood flows through the human brain at about 50 ml./100 g./min.; in an experiment with cerebral tissue in 100 vol. of fluid, in which initial substrate level and utilization *in vitro* equalled those *in vivo*, over 3 hr. would be required *in vitro* to decrease the added substrate concentration to its level in venous blood *in vivo*. In metabolic studies with separated tissues the volume of added fluid is rarely considered in relation to the duration of metabolism or to the metabolic rate of a tissue. When substrate may become limiting, the expedient of increasing its

concentration *in vitro* rather than increasing fluid volume is not often employed.

Accumulating products of *in vitro* metabolism may give more serious reason for addition of much fluid; few homeostatic devices are applied *in vitro* in contrast to the many *in vivo*. On the other hand, certain accumulation of products is normal and high fluid/tissue ratios greatly increase the time needed to establish *in vitro*, levels of products normal *in vivo*. Thus blood enters the brain 1·1 mM in lactate and leaves it at 1·28 mM (Gibbs, Lennox, Nims & Gibbs, 1942). Cerebral tissues placed in 100 vol. of glucose–saline have their lactate (about 15 mM) greatly reduced and require about 3·5 hr. to establish 1·28 mM lactate in the saline (see, for example, McIlwain & Grinyer, 1950). Constituents other than those formed from added substrates are also lost from tissue to fluid. Increasing fluid/tissue ratios probably increases such loss or the osmotic work necessary to oppose such dilution. Loss from cerebral tissues respiring in 50 vol. of glucose–saline was shown by a 30% fall in respiratory rate when the saline was replaced by a fresh specimen of the same initial composition (McIlwain, 1953).

In the present work, tissues have been studied with little or no added aqueous fluid. This has raised problems of provision of substrates and support for the tissue, for which answers have been sought by use of high substrate concentrations, non-aqueous solvents, and additional mechanical arrangements. We were encouraged in this by previous observations (McIlwain, 1951; McIlwain & Ochs, 1952) that the metabolism of cerebral tissues can remain normal while they are fixed in frames and after they have been bathed with liquid paraffin.

EXPERIMENTAL

General

Salines. The saline used in all experiments except those involving phosphate determinations contained: 134 mM-NaCl, 5·2 mM-KCl, 2·8 mM-$CaCl_2$, 1·3 mM-KH_2PO_4, 1·3 mM-$MgSO_4$, and 10·4 mM-Na_2HPO_4. Substrate when desired was added from a concentrated stock solution without diluting the saline by more than 3%. For preparation the following stock solutions were stored in the refrigerator: *A*, 0·725 M-NaCl, 0·029 M-KCl, 7·25 mM-KH_2PO_4 and 7·25 mM-$MgSO_4$; *B*, 0·11 M-$CaCl_2$; *C*, 0·1 M phosphate buffer (17·8 g. Na_2HPO_4 brought to pH 7·4 with N-HCl and diluted to 1 l.). To prepare 120 ml. of medium without precipitating Ca salts, 21·2 ml. of *A* was diluted to 106 ml. with glass-distilled water, 3·0 ml. of *B* added and the solution gassed with O_2 for 7 min.; 12·0 ml. of *C* were added slowly whilst gassing, after which gassing was continued for a further 10 min. When phosphates were to be determined the medium was buffered with aminotrishydroxymethylmethane (final concentration 0·025 M) in place of phosphate.

Experimental animals. For most experiments adult guinea pigs of 300–400 g. were used; they were stunned by a blow on the back of the neck and bled by decapitation before removal of the tissues. To obtain cerebral cortex slices of 100–120 mg. moist wt., the cerebral hemispheres were prepared as described by McIlwain (1951). Tissues from rats were obtained similarly; rabbits were killed by injection of air into the marginal ear vein.

Fresh tissue slices. Slices were cut from the freshly excised organ without the use of saline on blade or tissue. A narrow blade 10 cm. long and 2–3 mm. wide, held taut in a frame of silver-steel wire, was used and guided by a recessed glass slide to obtain slices of uniform thickness (approx. 0·35 mm.) After being cut, the slices adhered to the block of tissue and from this they were picked up by the wire rider of a torsion balance. In some cases they were transferred immediately with a spatula to a tared support (see below), for insertion into manometric vessels. In other experiments (see below) slices were cut with the minimum quantity of saline required to lubricate a blade of 15 mm. width and in these cases the slices adhered to the glass slide used as a guide. Any surplus saline was removed from the tissue immediately after cutting by placing the torn edge of a hardened filter paper at the side of the tissue as it lay on the slide.

Moist tissue slices. These were cut in the more usual fashion with blade and guide wet with the saline employed in the remainder of the experiment. After cutting they were floated from blade or guide to the experimental saline, and then drained from saline to a glass surface for weighing or further handling; in this condition they are referred to throughout as 'moist tissue'. Slices handled moist were mounted on their holders in a shallow dish of saline. They were floated as an open sheet above the holder which was held by forceps, and the holder lifted out of the saline so that it carried the slice with it. Excess saline was drained by placing on a pad of filter paper for 3–5 sec.

Supports for slices. A problem common to measurement of respiratory rates both with no added fluid and in non-aqueous media was that of providing adequate support for the tissue. Slices suspended in oil adhered to the vessel or to themselves, curling or forming a blob which could not be opened without damage to the tissue. The grid tissue-holders type *D* (McIlwain, 1951), devised previously as electrodes, were found very suitable for supporting the tissue in the present experiments. For moist tissue to which electrical pulses were not to be applied, a piece of metal gauze was used. The gauze should not be closely woven; silver wire gauze of 20-mesh was found suitable and gauze of stainless steel gave the same results.

Manometric vessels. These were vessels *A* (McIlwain, 1951) with a wide mouth (C24 standard socket) to admit the tissue-holders, and a displaced alkali-well to allow the holders to rest on the base of the vessel. They were conical, of 18–20 ml. volume, with a side arm with a valve-stopper.

Determinations

Lactic acid was determined according to Barker & Summerson (1941).

Inorganic and creatine phosphates. Methods described previously (McIlwain, Buchel & Cheshire, 1951) were modified as follows. The trichloroacetic acid extract (1 ml.) of the slice, centrifuged at 0°, was neutralized at 0° with N-NaOH (0·5 ml.; phenolphthalein) in a tapered centrifuge tube of 3–5 ml. Of a 10% w/v) solution of $CaCl_2$ saturated with $Ca(OH)_2$, 0·3 ml. was added and precipitation of the inorganic (and other) phosphates was allowed to proceed for 10 min. at room temp.; the tube was then centrifuged strongly.

For inorganic phosphate the precipitate, in its tube drained and wiped free from solution, was dissolved in 0·5 ml. 0·1 N-HCl and Berenblum & Chain's (1938) method applied on a small scale as described by Long (1943). Creatine phosphate was similarly determined in the supernatant or a measured portion, after adding 0·3 ml. of 10 N-H_2SO_4.

Measurements with fresh or moist tissue

A tissue holder carrying a slice was placed in a vessel already containing 0·15 ml. 5 N-NaOH in its well. A filter-paper wick was added to this and moist O_2 which had bubbled through 0·9% NaCl passed through the vessel for 7 min., after which it was placed in the thermostat and respiration followed in the usual manner.

Measurements with non-aqueous media

Fluids used. Light liquid paraffin, sp.gr. 0·835–0·850, was obtained from British Drug Houses Ltd. Silicone fluid D.C.200 was kindly supplied as samples of viscosities 0·65 centistokes (cS.), 1 and 5 cS. by Midland Silicones Ltd. Olive oil of B.P. (1948) specification was freshly obtained. In use, the liquids were equilibrated with the experimental saline and gas phases in the apparatus described below.

Measurement of solubility of O_2 in the fluids. The solubility is known to be high. Data relating specifically to the present experimental conditions were required for calculating manometric constants, and were obtained as described in the Appendix to this paper.

Preliminary experiments. Initial difficulties, apparently due to the high solubility of atmospheric gases in the non-aqueous fluids used, are illustrated by the following experiment. Light liquid paraffin (5 ml.), freshly gassed with O_2, was pipetted into a manometric vessel which was immediately placed on a manometer through which O_2 was passing. After 7 min. with O_2 flowing at room temperature the manometer and flask were put to the thermostat at 37° and shaken. After 5 min. the manometer tap was closed. Equilibrium was not reached until 40 min. later, and during this time 100 μl. of gas were gradually evolved. Only slight improvement was effected by passing the O_2 at 37° rather than at room temperature. The solubility of N_2 in the oil is such that when in equilibrium with air the volume used could contain about 400 μl. N_2, and it appeared that much N_2 had redissolved during pipetting.

Apparatus (Fig. 1). This was designed to equilibrate the non-aqueous fluid with the saline and gas employed, and to enable the pipetting to take place without exposure to air. The reservoirs *A* and *C* were permanently clamped in the thermostat used for manometric measurements. The manometric vessel *D* was temporarily held by a screw clamp in the position shown. The manometer *F* (described below) during initial procedures was fixed on the shaking arm of the thermostat by an additional clip on its back-board, which held the ground glass joint of the manometer about 2 cm. above the water level. Connexions from the gas cylinders to the reservoirs were of thick-walled rubber tubing; those from reservoir *C* to the vessel and manometer, of thin-walled surgical drainage tubing (approx. 2–3 mm. int. diam.). Oxygen from a cylinder was continually led through a two-holed rubber bung into the reservoir *A*, which contained the oil to be used in the experiment, in contact with a few ml. of saline. The pipette *B*, used for transferring the oil to the vessel, was a loose sliding fit in the other hole of the bung and when not in use was left in the position shown. Oxygen from the same cylinder also passed through the reservoir *C*, which contained oil and saline to saturate the gas with vapour; from here it was led either through the side-arm tap of the manometric vessel *D*, or through the three-way tap of the manometer *F*, depending on the position of the heavy 'bulldog' artery clip *E*.

Procedure. Before starting an experiment, O_2 was passed through both reservoirs for at least 30 min. to saturate the oil. Meanwhile, 0·15 ml. 5 N-NaOH was pipetted into the wells of all manometric vessels and tissue slices were cut and mounted. Vessels were then dealt with successively, the experimental ones first and an oil thermobarometer last. This contained only 5 ml. of oil from *A*. The holder (*G*) with its slice was fitted into an experimental vessel (*D*) which was immediately clamped in the thermostat and O_2 passed through the side-arm tap as shown in Fig. 1. Oil (5 ml.) from the reservoir *A* was rapidly pipetted into the vessel, which was then loosened from its clamp and attached to the appropriate manometer, already in position on the shaking arm, with its tap in position '*a*' and its right-hand limb filled with manometer fluid up to the tap. Concurrently with this last operation the clip *E* was moved from position 'i' to position 'ii' and the connexion to the vessel side-arm tap broken. Oxygen now passed into the flask via the manometer, which was lowered to its usual position, thus immersing the vessel. Gassing was now continued for a further 1·5 min., during which time the manometer and vessel were shaken at 40 oscillations/min. Finally the side-arm tap was closed and the manometer tap turned first to position '*b*' and then to position '*c*', and the vessel rubbed on to the joint. With two workers setting up an experiment consisting of four experimental vessels and one oil thermo-

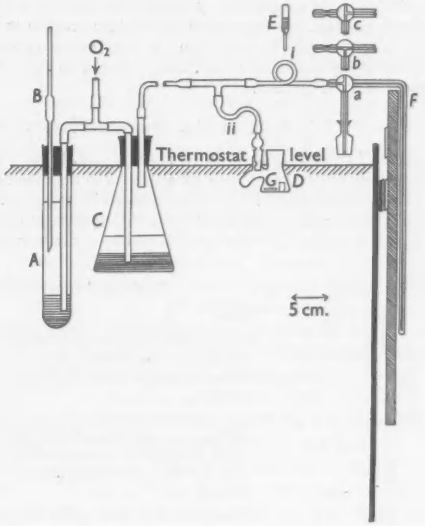

Fig. 1. Equilibration apparatus for manometric experiments in oils. Semi-diagrammatic. See text for description.

barometer, readings could normally commence 30–35 min. after killing the animal. The time taken to deal with one vessel was 3–4 min. and the moist tissue was never exposed to atmospheric air at room temperature for more than 15 sec. To complete the procedure in this time careful allocation of tasks was essential and involved overlapping in the preparation of successive slices and vessels.

In addition to the oil thermobarometer referred to above, a thermobarometer containing ungassed oil in atmospheric air was always included, as a check on the stability of the thermobarometer containing oil and oxygen. This should be placed at 37° at least 1 hr. before starting the experiment, in order to allow time for the oil vapour-pressure to become established. The rate at which O_2 was passed through the vessel and manometer was controlled by a flow meter (type FMO/V, Quickfit and Quartz Ltd.). A gas flow of 300–400 ml./min. was found adequate (and may have been excessive).

Design of manometers. The manometers used for most of these experiments were of an unconventional pattern illustrated in Fig. 1; they show some resemblance to the McGilvery-Machlett manometer described by McGilvery (1949). They were designed to overcome a difficulty encountered in using conventional manometers for experiments with the silicone fluids of viscosity 0·65 and 1 cS. These oils are appreciably volatile at 37°, with the result that when O_2 saturated with silicone vapour was passed through the manometer, some silicone condensed and formed a layer above the manometer fluid. With the new manometers gassing could be accomplished, as explained above, with the manometer fluid isolated from the gas. These manometers are more robust and flexible than conventional patterns and have been found very satisfactory for conventional procedures. They also possess certain advantages: for example the gas in the vessel can be shut off from that in the U-tube of the manometer by turning the tap to an intermediate position between those illustrated; further it is possible to evacuate the vessel on the manometer without disturbing the manometer fluid. The tap must be carefully greased and well ground.

RESULTS

Respiration of tissue without fluid

Tissues have been examined 'fresh' (cut and mounted without saline), 'moist' (cut with saline and mounted after floating in a dish of saline, but drained free from excess saline after mounting) and 'in saline' (the conventional procedure). In each case rates have been determined with and without glucose as substrate.

Initial rates of 'fresh' tissues. These rates proved surprisingly high; values with cerebral cortex are quoted in Table 1.

Rates with tissues which in their preparation had been left in saline were found to be lower because of two factors: (1) An apparently lower rate due to absorption of water before weighing; Table 1 shows this can amount to 20 % or more of the tissue weight, and such increase is adequate to explain the difference of respiratory rates (*c*) and (*d*) in Table 1. (2) An inherently lower rate of the tissue which had been exposed to saline. This is necessary to explain the difference between rates (*b*) and (*c*). It could be due to loss of material from the tissue to the saline or to dilution of tissue reactants by the absorbed saline.

Initial rates of 'moist' tissues. Such rates are compared in Table 2 with values for tissues immersed in about 50 vol. saline. All tissues had been handled in saline and their rates are thus lower than those of Table 1 (*a*)–(*c*) and of some previously recorded values (e.g. Wollenberger, 1947; see Cutting & McCance, 1946, and Kratzing, 1951).

Table 1. *Initial respiratory rates of 'fresh' cerebral cortex without added substrate*

Slices of guinea pig cerebral cortex 0·35 mm. thick were cut with a blade and guide without saline. Their fresh weight was determined and they were then treated as described in col. 2. Respiratory rates with specimens (*a*) were calculated on the basis of the fresh weight of tissue, and with (*b*), (*c*) and (*d*) on the basis of the last weighing described in col. 2. Conditions (*b*) imitated the cutting of slices with a blade moist with saline; conditions (*d*) imitated a commonly used procedure, followed in Table 2. Rates are followed by S.E. and number of observations in parentheses.

Specimen	Additional treatment	Respiratory rate (μmoles O_2/g./hr.)	Increase in weight (%)
(*a*)	None	104 ± 5·4 (7)	—
(*b*)	Slice moistened with saline on cutting blade; saline removed immediately afterwards and slice reweighed; then placed in manometric vessel	101 ± 3·0 (17)	1, 3
(*c*)	Slice moistened with saline on cutting blade; saline removed immediately afterwards and slice reweighed; subsequently slice left in saline 7 min. and then placed in manometric vessel	89 ± 1·7 (8)	1, 3
(*d*)	Slice moistened with saline on cutting blade, but removed immediately afterwards and slice weighed; left in saline 5 min., reweighed; returned to saline 2 min., then placed in manometric vessel	68 ± 5·9 (8)	20, 24

Table 2. *Initial respiratory rates of tissue slices with and without added saline*

Slices of guinea pig tissues were cut in the presence of saline and floated on to dishes of saline for mounting. Tissues weighed approx. 100 mg. and under conditions (*A*) were with 5 ml. saline while under (*B*) the adhering saline was about 0·09 ml. (see text). Values quoted are mean rates, followed by their S.E. and number of observations, for the period 15–45 min. after placing at 37·5°. Glucose when present was initially 10 or 33 mM.

Tissue	Glucose in saline	Respiratory rate (μmoles O_2/g. moist wt./hr.) (*A*) Tissue immersed	(*B*) Tissue moist
Cerebral cortex	–	40±1·2 (7)	44±1·7 (10)
Cerebral cortex	+	63±1·4 (9)	68±1·9 (10)
Kidney cortex	–	86±2·0 (14)	127±3·2 (12)
Kidney cortex	+	98±2·2 (14)	147±4·4 (6)
Liver	–	51±3·0 (7)	61±5·0 (6)

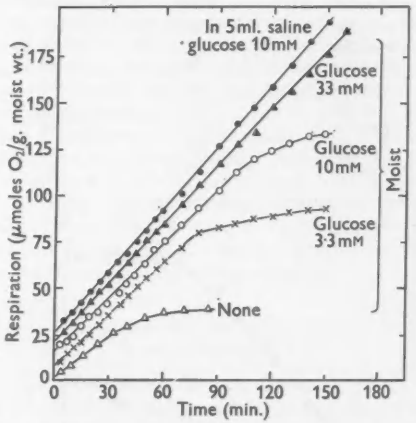

Fig. 2. Respiration of moist guinea pig cerebral cortex. Slices were cut in and floated into saline of glucose concentrations quoted. They were supported in manometric vessels in grid holders. The ordinates for slices respiring with glucose have been displaced upwards for clarity. The greater respiratory rate of the moist tissue, seen in the average values of Table 2, is not evident in the particular experiment illustrated here.

The average respiratory rates of tissues respiring moist were in every case greater than those of tissues immersed in saline. The difference was especially notable with kidney cortex, when rates with glucose were half as great again when the volume of saline was decreased to 0·002 of the normal amount. The presence of substrate afforded higher rates with the cerebral and renal tissues (it was not examined with liver), in spite of the small quantity of nutrient saline involved.

In some initial experiments with moist cerebral cortex and liver, 0·35 mm. slices were laid on filter paper moist with glucose salines on the base of conical vessels. The tissues were found to respire at rates some 10–15% lower than those of Table 2, presumably due to poor oxygenation.

Maintenance of respiration. Rates such as those of the fresh tissue in Table 1 decreased after about 30 min. This occurred also with moist tissues prepared in absence of substrate, but with substrate respiration could remain high for some 2 hr. (Figs. 2 and 4). With cerebral cortex in saline the effect of added glucose in increasing respiratory rate could be seen in the first few minutes of metabolism. This was evident also with the moist tissue which had received glucose only from the saline in which it has been prepared. The glucose so provided is limited, and higher concentrations of glucose were found necessary to maintain respiration after the first hour, than was the case when the relatively large volume of saline was provided in normal metabolic practice (Fig. 2). However, by preparing the tissue in saline with 33 mM glucose in place of the usual 10 mM, respiration remained high for at least 2·5 hr.

Respiration of slices bathed with non-aqueous fluids

Certain of the functions of aqueous suspending media in metabolic experiments may possibly with advantage be carried out by non-aqueous media. To examine this, respiratory rates of tissues in saline have been compared with those of moist tissues supported and immersed in liquid paraffin, olive oil or silicone fluid which were saturated with oxygen and saline as described in the experimental section.

Initial rates of respiration. Guinea pig cerebral cortex was examined in all three media. In liquid paraffin and olive oil respiratory rates were not significantly different from those found by conventional procedures in saline. With silicone fluid as suspending agent a rate was obtained 10 or 12% higher than that in saline ($P < 0·05$) or in liquid paraffin ($P < 0·01$). Other tissues from other animal species gave results summarized in Table 3.

Maintenance of respiration and provision of substrate. Unless additional substrate was provided, respiratory rates of moist tissue in oil fell after about 30 min. at 37° (Fig. 3). However, it was again easy to provide substrate by preparing the tissue in saline containing 33 mM glucose.

Table 3. *Initial respiratory rates of moist tissue slices bathed with non-aqueous media*

Slices were prepared in glucose salines except those marked * which were without substrate. All were mounted in tissue-holders type *D* (McIlwain, 1951). When sufficient values are available the mean rate is followed by its S.E. and the number of observations. The initial rate refers to the period 20–40 min. after placing at 37·5°. The respiratory rates in saline were obtained in the same experiment, or in experiments conducted at about the same time as the rates recorded in non-aqueous fluids.

		Moist tissue in non-aqueous fluid		Respiratory rate of tissue in saline (μmoles O_2/g. moist wt./hr.)
Animal	Tissue	Fluid	Respiratory rate (μmoles O_2/g. moist wt./hr.)	
Guinea pig	Cerebral cortex	Silicone	68·6±2·01 (12)	62·9±1·37 (9)
	Cerebral cortex	Paraffin	59·8±1·35 (6)	
	Cerebral cortex	Olive oil	60, 62	—
Rabbit	Cerebral cortex	Paraffin	40, 40	48, 52
Guinea pig	Kidney cortex	Paraffin	108	110
	Liver	Paraffin	67	66
	Spleen	Paraffin	50	47
Rat	Liver	Paraffin	63	73
	Spleen	Paraffin	56	66
	Diaphragm	Paraffin	51	59
Guinea pig	Kidney cortex*	Paraffin	112	98
	Liver*	Paraffin	62	40

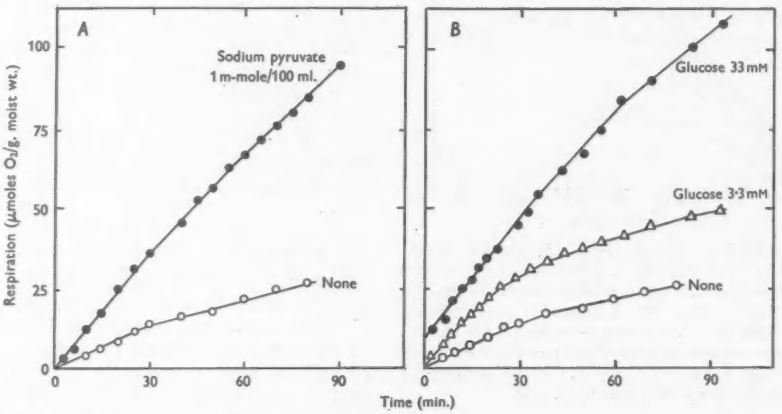

Fig. 3. Respiration of moist guinea pig cerebral cortex immersed in silicone fluid. Slices cut and floated into salines and supported in manometric vessels in grid holders. *A*: substrate (sodium pyruvate) supplied by 'homogenization' in oil in quantity quoted (preparative saline contained no substrate). *B*: substrate supplied by incorporation in preparative saline at concentrations quoted (the ordinates for the slices respiring in glucose have been displaced upwards for clarity).

An estimate of the amount of substrate available to slices of guinea pig cerebral cortex from the small quantity of preparative saline remaining associated with the slice was obtained in one series of experiments by weighing. From the weight of the tissue-holder plus moist slice, the original moist weight of the slice and the weight of the holder, the weight of saline transferred to the manometric vessels was calculated. The average for six slices, whose weights ranged from 100 to 150 mg. was 90 mg., range 73–117 mg. However, a proportion of this saline was obviously adherent to the tissue-holder and some of this was observed to be washed off subsequently by the oil in the vessel. A determination of amount of saline which remained associated with the slices themselves at the end of a 2 hr. experiment in liquid paraffin was therefore made. This was done by removing the slices from their holders and weighing them together with adherent oil and saline. The aqueous phase was then removed by drying in a vacuum desiccator and the slices were reweighed. From the difference in these two weights and the dry weight of the tissue (found to be 19 % of its original wet weight) the weight of the remaining

saline was found to average, for 6 slices, 15–20 mg. In these experiments the substrate in the saline was 33 mM glucose. Taking from the above determinations a figure of 0·05 ml. as representing the average volume of saline in contact with the slices during the experiments, it follows that 1·7 μmoles of glucose are available to the tissue from this source. The oxidation of this glucose would require 10·2 μmoles oxygen. The mean oxygen consumption by the tissues in the 2 hr. before their oxygen uptakes began to fall was in fact 10 μmoles. This agreement doubtlessly is partly coincidental, for no allowance has been made for endogenous substrate nor the possibility of preferential assimilation of glucose from saline before the slice was transferred to the vessel. The values, however, show that appreciable quantities of substrate can be made available for metabolism in a very much smaller bulk of saline than is normally employed.

The non-aqueous fluid has also been used as a vehicle for providing substrate. Sodium pyruvate when ground in oils in an all-glass homogenizer afforded a suspension from which it was only slowly washed by shaking with water. Such a suspension in silicone was used as substrate in the experiment of Fig. 3 and is seen to be actively oxidized.

Exchange, removal, or addition of fluid to respiring slices

These procedures enable the effects of different conditions to be examined on the same specimen of tissue. As shown in Table 4 and Fig. 4, the greater part of the saline may be removed from slices of kidney and cerebral cortex respiring under conventional conditions and the tissue continues to respire with little change in rate. When succinate was provided in the first period as substrate for cerebral tissues, the rate was lower in the second period but this is the case whether or not the medium has been removed.

On the other hand, when saline was added to moist tissues, their respiratory rates invariably fell. This is shown to be the case with cerebral cortex in Table 4; the fall was greater with kidney cortex (Fig. 4; Table 4) and took place whether or not glucose had initially been provided in the saline used for preparing the tissue.

Application of electrical pulses to tissues

One of the aims of the present studies was to obtain conditions under which cerebral tissues might respond to applied electrical pulses (see McIlwain, 1951) in the absence of much saline. When electrical pulses adequate for metabolic response in saline were applied to moist tissue or tissue in oil, increase in respiration was usually found, but the increase was small. Larger and more regular response was obtained when the slices were first incubated under conventional conditions. The aqueous medium was then removed and the slice left moist or with different oils. Electrical pulses of characteristics adequate for response in salines (McIlwain, 1951, 1954) were then also effective when the tissue was moist or when it was surrounded by liquid paraffin (Table 5 and Fig. 5). No response was given in silicone or olive oil.

Addition of inhibitory and stimulating agents to moist tissue and tissue in liquid paraffin

Inhibitory substances could be added satisfactorily in liquid paraffin. It was especially interesting to examine substances which in saline showed inhibition of respiration in presence of applied electrical pulses though not in their absence. Table 6 shows that such effects were observed with oil containing atropine at 1 mM but not with atropine at 0·1 mM. When atropine was incorporated

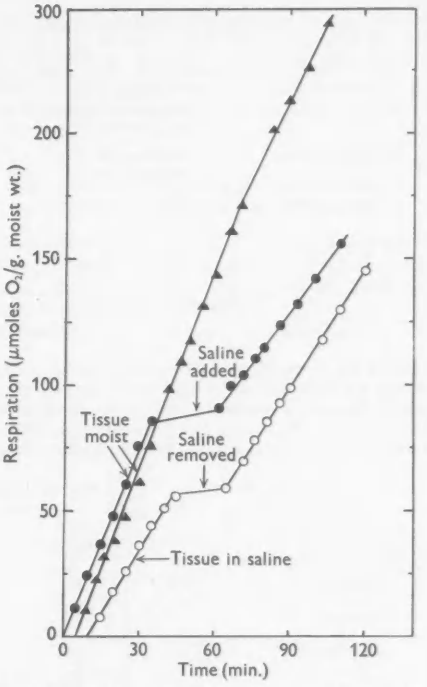

Fig. 4. Respiration of moist guinea pig kidney cortex. Slices cut and floated into saline containing 10 mM glucose, and supported in vessels in grid holders. Saline (5 ml.) was added and removed from vessels where indicated. (The abscissae for two slices have been displaced to the right for clarity.)

Table 4. *Respiratory rates of tissue slices before and after exchange, removal or addition of fluid*

Guinea pig tissues were prepared as in Table 2 and were mounted in tissue-holders. Fluid volumes were 5 ml. except in the first part of Expt. *B*, when the fluid adhering to the tissues was about 0·09 ml. Replacement or removal of fluid occupied 2–6 min., during which time the tissue was at room temp.

Expt. *A*. Removal of saline or its replacement by non-aqueous media

	First 30 min.		Second 30 min.	
Tissue	Substrate in saline (mM)	Respiratory rate (μmoles O_2/g. moist wt./hr.)	Non-aqueous medium or state of tissue	Respiratory rate (μmoles O_2/g. moist wt./hr.)
Cerebral cortex	Glucose, 13·3	64, 62	Silicone	86, 66
	Glucose, 33	72, 75, 68	Silicone	70, 78, 79
	Glucose, 33	70, 76, 74	Olive oil	65, 63, 59
	Glucose, 33	68, 74	Paraffin	65, 76
	Glucose, 33	65, 79, 77	Saline removed and moist tissue left supported in vessel	69, 73, 69
	Na glutamate, 50	90, 82		83, 82
	Na pyruvate, 50	79, 73		81, 82
	Na succinate, 50	100		64
Kidney cortex	Glucose, 10	102, 94	As above	96, 94
	None	98, 98	As above	90, 90

Expt. *B*. Addition of saline after a period of respiration with no fluid

	First 30 min.		Second 30 min. with added saline	
	Substrate in preparative saline (mM)	Respiratory rate (μmoles O_2/g. moist wt./hr.)	Substrate in added saline (mM)	Respiratory rate (μmoles O_2/g. moist wt./hr.)
Cerebral cortex	Glucose, 33	60, 66	Glucose, 33	44, 48
	Glucose, 10	148	Glucose, 10	78
Kidney cortex	None	125, 115	None	84, 84
	None	128	None	78

Table 5. *Application of electrical pulses to tissue respiring moist or in non-aqueous media*

Guinea pig cerebral cortex slices were prepared in saline with 33 mM glucose and allowed to respire for 30 min. in this medium before its removal or replacement by non-aqueous media. Respiratory rates are not quoted for this preliminary period. Alternating condenser pulses were applied at 100/sec. and were of other characteristics described in the table. When sufficient values are available the mean rate is followed by its S.E. and the number of observations, and the increase in respiration is calculated as the difference between each group.

	Electrical pulses applied		Respiration during second period (μmoles O_2/g. moist wt./hr.)		Increase with electrical pulses (%)
Medium during second period	Peak potential (v)	Time constant (msec.)	Unstimulated	Stimulated	
Silicone	18	1·0	77 ± 2·3 (5)	81 ± 1·9 (5)	5
Olive oil	18	1·0	60, 62	66, 63	5
Paraffin	18	1·0	66 ± 1·7 (8)	88 ± 3·0 (8)	34
	12	1·0	75	95	27
	12	0·25	65	82	26
	24	1·0	75	105	40
	24	0·25	65	88	35
None; tissue supported moist in vessel	18	1·0	67 ± 2·0 (14)	98 ± 2·1 (14)	48

in saline at these concentrations, and the saline removed leaving the moist tissue, comparable inhibition was not found. The oil was thus performing a valuable function in maintaining a supply of the inhibitor.

Cocaine or butobarbitone added to saline in which slices were respiring did however affect their subsequent behaviour after the saline had been removed leaving the moist tissue. Applied electrical pulses normally increasing respiration then had very little effect.

The use of a non-aqueous medium enabled oil-soluble agents to be added from side arms during metabolism. 2:4-Dinitrophenol, added to form a 50 μM solution in the oil, greatly increased the respiration of moist cerebral tissue and electrical pulses were then ineffective (see McIlwain & Gore, 1951). Phenobarbitone and butobarbitone added during electrical stimulation inhibited the respiratory response (Fig. 5).

Lactic acid formation

In early experiments, measurements were made of the pH of ground suspensions of cerebral cortex slices after periods of respiration moist or in non-aqueous media, using bromothymol blue. With slices prepared in saline containing glucose at 10 mM or less, the pH of the suspension after O_2 uptake had fallen off (e.g. Fig. 2) was invariably about 7·0. With 33 mM glucose in the preparative saline the pH was about 6·0 after 2 hr. of respiration. This suggested some accumulation of acid metabolites, amongst which lactic acid might be expected to preponderate.

Lactic acid appeared in the saline or slice in absence of added substrate when guinea pig tissues were examined (Table 7; see also McIlwain, 1953). The amount from the moist tissue was smaller in total amount but higher in concentration than with the tissue in saline. This was true also when glucose was added as substrate. Lactic acid in the moist tissue with or without liquid paraffin appeared to reach a maximum level of about 7–9 μmoles/g. when 10 mM glucose was provided and metabolism progressed for less than 1 hr. Metabolism from 33 mM glucose or for 100 min. did not greatly increase this quantity. Previous experiments have shown that 6·7 mM or higher concentrations of lactic acid in saline increase respiration of cerebral tissues while 2 mM does not (McIlwain, 1953). Probably such further oxidation limits the level of lactic acid attained in the present experiments. This also makes understandable the greater quantity of lactic acid which accumulates in the saline under conventional manometric conditions. The last column of Table 7 gives an estimate of the percentage of the glucose metabolized, which has been converted into lactic acid. The calculation depends on a previous finding (McIlwain, Anguiano & Cheshire, 1951) that the glucose not so converted is oxidized completely. The proportion oxidized is greater with minimal fluid.

Phosphocreatine and inorganic phosphate in cerebral cortex with minimal saline

The phosphocreatine and inorganic phosphate levels of cerebral tissues *in vivo* are grossly disturbed during excision and preparation of the brain for

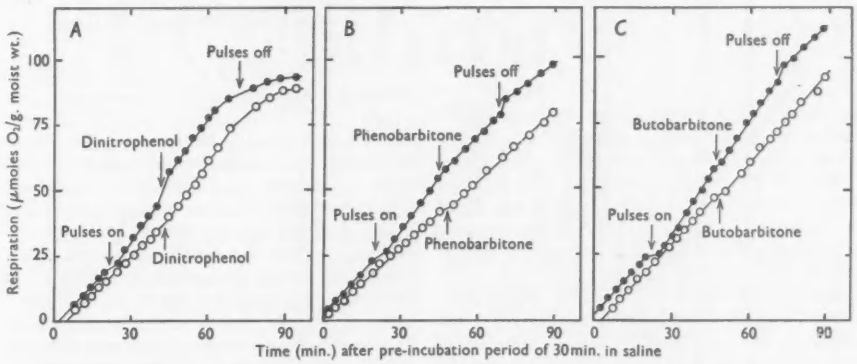

Fig. 5. Stimulation and inhibition of respiration of moist guinea pig cerebral cortex tissue immersed in liquid paraffin. Slices were cut and floated into saline containing 33 mM glucose, mounted in grid holders and allowed to respire in this saline for 30 min., before its removal and replacement by oil. Alternating condenser pulses, 100/sec., 18 v peak potential and time-constant 1 msec. were applied where indicated. *A*: effect of 50 μM 2:4-dinitrophenol on stimulated and unstimulated respiration; *B*: 0·1 mM phenobarbitone; *C*: 0·1 mM butobarbitone. Added substances were initially in side arms in paraffin at 10 times the concentrations quoted, and tipped as indicated.

Table 6. *Action of inhibitory agents on stimulated respiration of moist tissue and tissue respiring in liquid paraffin*

Guinea pig cerebral cortex slices were prepared in salines with 33 mM glucose. Electrical condenser pulses were alternating, of frequency 100/sec., peak potential 18v and time-constant 1·0 msec.

First period		Second period		Third period (medium as in second period)		Increase in respiratory rate in third period (% of that in second period)
Substance added to glucose saline (mM)	Respiratory rate (μmoles O_2/g. moist wt./hr.)	Medium	Respiratory rate (μmoles O_2/g. moist wt./hr.)	Agents (mM)	Respiratory rate (μmoles O_2/g. moist wt./hr.)	
None	68, 66, 61	None	68, 66, 60	Pulses	104, 100, 104	53, 51, 73
Atropine, 0·3	64, 65	None	57, 54	Pulses	80, 93	36, 67
Atropine, 1·0	70, 72, 72, 62, 64	None	67, 64, 65, 57, 62	Pulses	93, 95, 82, 81, 83	38, 48, 26, 42, 34
Butobarbitone, 1·0	64, 53	None	48, 51	Pulses	51, 54	6, 6
Cocaine, 1·0	60	None	58	Pulses	62	7
None	65	Paraffin	69	Pulses	100	45
None	70, 74	Paraffin with 0·1 mM atropine	60, 67	Pulses	92, 83	53, 24
None	64, 68	Paraffin with 1 mM atropine	63, 56	Pulses	64, 61	1, 9
None	62	Paraffin	59	2:4-Dinitrophenol, 0·025	89	50
None	69	Paraffin	68	Pulses	94	38
				Pulses + phenobarbitone, 0·1	75	10
None	63	Paraffin	67	Pulses	90	34
				Pulses + butobarbitone, 0·1	79	18

Table 7. *Lactic acid formation by cerebral cortex slices respiring moist, in liquid paraffin and in saline*

Guinea pig cerebral cortex slices (except * which were from rabbit) in grid holders were incubated at 37·5° for 90–100 min. At the end of this period the tissue was ground in 16 ml. 4% (w/v) $CuSO_4$ to which washings or samples from the vessel and holder were also added, and lactic acid was determined in the suspension after adding $Ca(OH)_2$ and centrifuging, according to Barker & Summerson (1941).

Tissue (mg. moist wt.)	Medium	Saline in vessel: Volume (ml.)	Saline in vessel: Glucose (mM)	Oxygen uptake (μmoles)	Lactic acid formed (μmoles)	$\frac{\text{Lactate} \times 100}{\text{Lactate} + \frac{1}{3}O_2}$ (see text)
80	Saline	5	0	3·1	0·39	—
70	Saline	5	10	7·7	3·9	60
103	Saline	5	33	10·2	5·4	62
105	None	Approx. 0·1 ml./ 100 mg. adhering to tissue	0	6·0	0·18	—
106	None		3·3	10·1	0·17	5
83	None		10	8·3	0·51	16
80	None		33	10·4	0·56	14
83	Paraffin	Approx. 0·05 ml./ 100 mg. adhering to tissue	33	4·9	0·63	27
72	Paraffin		33	6·6	0·56	20
86*	Paraffin		33	5·7	0·69	27
72*	Paraffin		33	4·6	0·67	30

Table 8. *Inorganic and creatine phosphates in guinea pig cerebral tissues*

Tissues under conditions *A* were on silver gauze and in *B* and *C* were in grid holders. After incubation, tissues *A* were washed from the gauze with trichloroacetic acid to homogenizers and *B* and *C* were transferred rapidly to trichloroacetic acid in homogenizers. Values given are each the mean of 2 or 3 results which were within ±8% of the mean.

Condition of tissue when incubated	Time of incubation (min.)	Phosphocreatine (μmoles/g.)	Inorganic phosphate (μmoles/g.)
A. Fresh; no previous contact with saline	0	0·49	17·1
	15	0·51	15·9
	30	0·20	17·7
B. Moist; previously floated in saline with 0·01 M glucose	0	0·39	5·9
	30	0·81	5·7
	60	0·50	8·2
B. Moist; previously floated in saline with 0·033 M glucose	0	0·66	6·1
	30	0·98	6·1
	60	1·09	6·2
C. In saline with 0·01 M glucose	60	1·71	3·1
C. In saline with 0·033 M glucose	60	1·63	2·9

metabolic experiments, but are largely restored by incubation in glucose salines (McIlwain, Buchel & Cheshire, 1951).

In fresh tissue incubated without contact with salines or substrates, phosphocreatine remained low and inorganic phosphate high (Table 8). Thus endogenous substrates here, as in experiments with saline, support respiration but not maintenance of the phosphate levels. Slices prepared in glucose-containing salines were found to have more normal levels of phosphocreatine and inorganic phosphate, but values tended to be erratic. However, after restoring the creatine phosphate by incubation in glucose-containing salines, it was well maintained in the moist tissue which retained only some 0·1 ml. fluid/0·1 g. tissue. With 30 mM glucose during pre-incubation, phosphocreatine levels fell by no more than 15% in 15 min., though with 10 mM glucose the fall was of 38% in 30 min. (Fig. 6).

DISCUSSION

Present observations bridge a gap between two methods of handling animal tissues. For observing mechanical or electrical response, intact muscle and nerve have often been studied in moist gas but with little or no added aqueous phase. With these tissues, such conditions may suffice for respiratory measurements (Meyerhof & Schulz, 1927; Gerard, 1932). They have also been employed with the cornea (Langham, 1952). Yet with tissue fragments whose cut surfaces increase likelihood of loss, a tradition of suspension in much fluid was early established. Warburg (1923, 1924) and Minami (1923) used 30–800 vol. of saline with liver and carcinoma specimens, and Dixon (1951) suggests 60–400 vol. Warburg (1915, 1924) measured respiration at different fluid/tissue ratios with spermatozoa and with tissue slices, but considered only two or three-

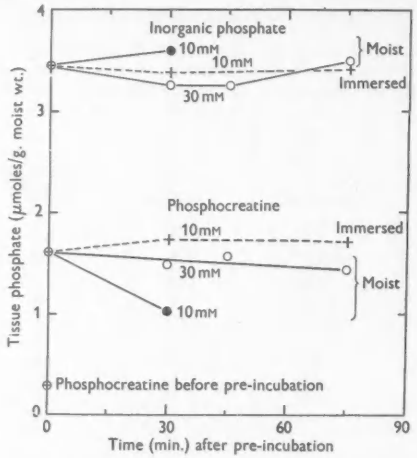

Fig. 6. Maintenance of creatine phosphate and inorganic phosphate in slices of guinea pig cerebral cortex cut in salines of the glucose concentrations quoted in the figure. All slices were initially incubated in 5 ml. of saline for 30 min., after which the saline was removed from the vessels indicated and their incubation continued for 30, 45 or 75 min.

fold variation in ratios 100–400 in value. Dixon (1951) pointed out discrepancies which could arise especially in muscular tissues when suspensions probably already in much fluid were diluted three-fold, but did not raise the question of the general use of high fluid/tissue ratios.

The studies of the present paper indicate that many metabolic observations may be made with sliced tissues from brain, kidney, spleen and liver with volumes of saline no greater than those of the tissues themselves. This is 0·002–0·01 of the conventional quantity, but was adequate to supply substrates and maintain respiration at 37° for 1–2·5 hr. Change in composition of this saline brought about changes in behaviour of the tissue. Moreover, in the case of cerebral cortex, creatine phosphate could be adequately maintained in the moist tissue. The level of creatine phosphate in the brain *in situ* is very susceptible to any failure in supply of materials from the bloodstream and in the separated tissue in saline is promptly lowered if oxygen and glucose are not supplied or if inorganic constituents of the medium are much altered from normal (McIlwain, 1952). Hence its maintenance in the moist tissue gives further indication that such conditions are metabolically satisfactory. Also contributing to this impression and possibly interconnected with the maintenance of creatine and inorganic phosphates are the observations that respiration of the tissue can be increased by electrical pulses and by 2:4-dinitrophenol. This stimulation implies that the restraint or control imposed on the level of respiratory (and other) metabolism in the cell-containing tissue in saline is also reproduced in the moist tissue.

Apart from these similarities which indicate conditions with little saline to be not inferior to those with excess, special interest attaches to circumstances when little saline may be advantageous. Thus respiratory rates were in some cases higher with minimal saline. Oil-soluble substances were supplied to the tissue. Substrate utilization may more closely resemble its *in vivo* course. An example of this is seen in the formation of lactic acid from glucose by cerebral tissues. *In vivo* about 15% of the glucose metabolized by the brain is found by arterial-venous difference to be converted to lactic acid, and almost all the remainder oxidized completely (Himwich & Himwich, 1946). Under conventional conditions *in vitro* some 60% or more of the glucose appears as lactic acid (McIlwain & Grinyer, 1950; McIlwain, Anguiano & Cheshire, 1951), and many agents increase the proportion so appearing. With moist tissue alone or in oil, assuming lactic acid and CO_2 to be the only products, the fraction appearing as lactic acid was 15–30% (Table 7). Reasons were given above for considering the artificially high formation of lactic acid under conventional conditions to be due to its dilution in the large quantity of aqueous fluid supplied. Possibly one reason for the frequent use of large fluid volumes lies in their acting in this way as trapping agents for intermediary metabolites, a situation which offers analytical advantages although limiting quantitative comparison with the behaviour of the tissue *in vivo*.

SUMMARY

1. Respiration of sliced cerebral cortex, kidney cortex, liver and spleen from guinea pig, rat, or rabbit has been measured in the presence of minimal aqueous fluid. The tissue rested on a grid in apparatus designed for these procedures.

2. Tissues examined fresh or moist (prepared in saline but drained from it before use) respired at rates initially similar to or higher than those under conventional conditions which employed 100–1000 times as much aqueous phase. Rates subsequently fell unless substrate was provided.

3. Comparably high respiratory rates were observed with the tissues immersed in a silicone fluid, light liquid paraffin, or olive oil.

4. Substrate was provided either during preliminary preparation of the tissue, or in a non-aqueous phase during metabolism.

5. Inhibitory and stimulating substances were also effective when so added. Respiration of

moist cerebral cortex was increased by electrical pulses and the increase opposed by certain added agents.

6. Formation of lactic acid by cerebral tissues in minimal aqueous fluid was closer to its normal *in vivo* value than was its formation with the conventional excess of saline.

7. A considerable level of phosphocreatine could be maintained in moist cerebral tissues for at least 75 min., while they respired at 37° in oxygen.

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APPENDIX

Manometric Determination of the Solubility of Oxygen in Liquid Paraffin, Olive Oil and Silicone Fluids

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For the calculation of manometric vessel-constants for experiments conducted in non-aqueous fluids, the solubility of oxygen in the medium at the temperature of the thermostat was required.

The manometric apparatus of Van Slyke & Neill (1924) is not convenient for measurements at temperatures above that of the laboratory; the present method employs a conventional thermostat and shaking apparatus and involves measuring the entry of oxygen into the de-gassed oil; by this technique information can also be obtained about the rate at which the gas dissolves in the oil. A method somewhat similar in principle but for larger volumes especially of solid fats which have lower vapour pressures and in which gases are less soluble than the present oils, has recently been described by Davidson, Eggleton & Foggie (1952).

EXPERIMENTAL

The essential part of the apparatus is illustrated in Fig. 1. The oil reservoir *A* contained, besides the oil, anhydrous Na_2SO_4 and was mounted in the thermostat. Before a determination, O_2 (dried by passing over P_2O_5) was bubbled through the reservoir and out into the atmosphere via the three-way stop-cock *B*. Semi-pressure tubing (ext. diam. 8 mm.; int. diam. 3 mm.) connected the manometer *C* (Rodnight & McIlwain, 1954) via the T-piece *D* to a high-vacuum pump and also to *B*. The special manometer vessel *E*, of approx. 30 ml. total capacity, was fitted with a stop-cock (5 mm. tap bore and greased with high-vacuum rubber grease) leading to a side chamber; the latter, together with the tap bore, had a capacity of 1·5 ml. 2 ml. of oil were pipetted into the vessel, which was placed on the manometer cone (greased with silicone high-vacuum grease) and immersed in the thermostat. With the manometer stop-cock in the position illustrated in Fig. 1, the pump was switched on

and evacuation to remove gas dissolved in the oil continued for 15–20 min. (shorter times were used for silicone fluid of viscosity 1 cS.). At the end of this time the manometer and vessel were tipped so as to fill the side chamber with de-gassed oil and the stop-cock was closed. The pump was then isolated by closing the stop-cock F and warm O_2 carrying oil-vapour from A admitted to the vessel by turning stop-cock B first to position $B1$ and immediately afterwards to position $B2$. The manometer stop-cock was next turned to connect the vessel to the manometer limbs, shaking at 110–120 oscillations/min. commenced and manometer readings taken every 2 or 3 min. Pressure changes were recorded until the oil remaining in the main part of the vessel was saturated with O_2 and had begun to establish a vapour pressure at a steady rate (A–B, Fig. 2). Then the stop-cock F was opened and the de-gassed oil flowed into contact with the gas phase. Readings were continued every min. until a steady rate was again established (B–C, Fig. 2). Changes in atmospheric pressure during the experiment were corrected by a thermobarometer containing air only. In some experiments the flask was weighed before and after the estimation to determine whether any loss of oil had occurred during evacuation; no losses were found with liquid paraffin, olive oil or with silicone fluids of viscosity 5 cS. or greater; losses occurred with the silicone fluid viscosity 1 cS. and this was allowed for in the calculation.

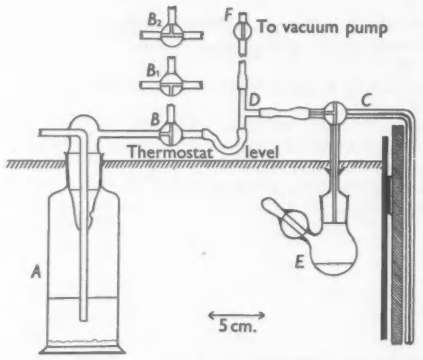

Fig. 1. Manometric apparatus used for the determination of the solubility of O_2 in non-aqueous fluids. For description see text.

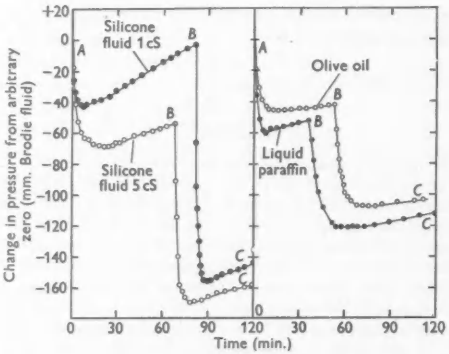

Fig. 2. Pressure changes on addition of de-gassed oils to oxygen atmospheres. At zero time A the de-gassed oil is isolated in the side chamber of the vessel and the remainder of the vessel filled with O_2. After pressure changes become regular the de-gassed oil is allowed to run into the vessel at B by turning a stop-cock and the resulting fall in pressure measured.

Typical curves illustrating the above procedure for the four oils examined are given in Fig. 2. The total change in pressure on adding the de-gassed oil was calculated by extrapolation of the rate at which the oil was evolving its vapour. Extrapolation could be avoided by waiting several hours for steady values and this procedure was found to give the same results.

The solubility of O_2 in the oil was calculated from the following considerations: Let V_g = vol. of vessel not occupied by oil; V_1 = total vol. of oil added to vessel; $V_2 = V_1$ less vol. of oil in side chamber; T = temp. of thermostat (° K); P_0 = standard pressure of 760 mm. Hg; P = pressure in vessel immediately before addition of de-gassed oil, being atm. pressure plus the difference in pressure registered in the manometer; h = change in pressure on addition of de-gassed oil (corrected); α (Bunsen coefficient) = ml. of gas (at s.t.p.) dissolved by 1 ml. of oil at temp. T and under a partial pressure of 760 mm. Hg.

Initially the O_2 in the vessel comprises that in the gas phase, $V_g\ 273P/TP_0$, and that in the oil phase, $V_2\alpha P/P_0$. At the end of the experiment these values are:

$$V_g\ 273(P-h)/TP_0 \quad \text{and} \quad V_1\alpha(P-h)/P_0.$$

As the total amount of gas remains unchanged it follows that

$$V_g\ 273\ P/TP_0 + V_2\alpha P/P_0 = V_g\ 273(P-h)/TP_0 + V_1\alpha(P-h)/P_0$$

and

$$\alpha = hV_g\ 273/T[P(V_1-V_2)-hV_1].$$

RESULTS AND DISCUSSION

Values for the solubility of oxygen in liquid paraffin, olive oil and silicone fluids of viscosities 1 and 5 cS., as determined by this method are given in Table 1. The figures for silicone fluids are surprisingly high.

Table 1. *The solubility of oxygen in liquid paraffin, olive oil and silicone fluid*

Temperature, 38°; αO_2 (38°) = ml. of O_2 (at s.t.p.) dissolved by 1 ml. of oil at 38° and under a partial pressure of 760 mm. Hg. Where sufficient data is available mean values are quoted followed by the standard deviation and the number of determinations in parentheses.

Oil	αO_2 (38°)
Liquid paraffin (sp.gr. 0·835)	0·098 ± 0·0017 (5)
Olive oil, B.P. (1948)	0·102 ± 0·0061 (6)
Silicone fluid (viscosity 5 cS.)	0·251, 0·250, 0·210
Silicone fluid (viscosity 1 cS.)	0·304, 0·297

The scale of the method limits its accuracy to ±5% in a single determination, which is adequate for determinations of solubilities required for calculating manometer-vessel constants. Greater precision can be obtained by repeated determinations (see Table 1) and by increasing the dimensions of the apparatus. The special advantage of the present method compared with that of Davidson *et al.* (1952), lies in the fact that the initial pressure in the vessel is accurately measured, rather than estimated by extrapolation. In addition, temperature equilibration is ensured, as preliminary separation of the oil allows time for it to regain the temperature of the thermostat after cooling which occurs during evacuation.

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Electrical Pulses and the Metabolism of Cell-free Cerebral Preparations

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Metabolic response of sliced or chopped cerebral tissues to electrical pulses has shown several characteristics relating it to electrophysiological properties of the central nervous system (McIlwain, 1951*a*; 1954). Many of these properties can be interpreted as dependent on movements of ions to and from cells (see, for example, data collected by Eccles, 1953).

Certain changes in respiration and phosphorylation in cell-free preparations with applied electrical pulses (Abood, Gerard & Ochs, 1952) have been considered similar to those previously found in cell-containing systems. Responses in the two types of preparations have now been examined in parallel, and considerable differences found between them.

EXPERIMENTAL AND RESULTS

Particulate preparations

Glycolytic and respiratory responses to electrical pulses were less when cerebral tissues were very finely chopped and were doubtful or absent in 'homogenized' tissue, examined under conditions otherwise similar to those of the experiments with slices (Table 1*A*).

Media suitable for metabolism of sliced tissue are not optimum with cell-free preparations. In particular, in view of the connexion between changes in respiration, glycolysis, and phosphate derivatives in sliced cerebral tissue (McIlwain, 1952) it appeared desirable to examine preparations capable of oxidative phosphorylation. Two sets of conditions were chosen, using suspensions of the whole tissue, and a mitochondrial preparation.

Suspensions. In early experiments using electrodes of electrodeposited gold, partial but erratic inhibition of both respiration and phosphorylation was observed. Artifacts have previously been traced to the metal of the electrodes (McIlwain, 1951*a*) and molybdenum is free from many such effects (Ayres & McIlwain, 1953). Potential loss at molybdenum–saline interfaces is somewhat greater than at gold–or silver–saline interfaces, but full metabolic response in slices is readily obtained (Table 1*A*). Vessels with molybdenum electrodes were therefore used for examining effects of applied electrical pulses on suspensions under conditions in which phosphorylation was actively proceeding at some 200–300 μatoms P esterified/g. tissue/hr., with P/O ratios (atom/atom) of 1·2–1·8. Under these circumstances, pulses which with slices increased respiration twofold and glycolysis threefold, with suspensions increased oxygen uptake slightly (mean, 14%) and phosphate esterification to nearly the same degree (10%). The P/O ratio thus appeared to fall very slightly. This effect was extremely small in comparison with that which can be brought about by an agent such as 2:4-dinitrophenol (Table 1*B*) in the suspensions or by comparable pulses on the phosphates of sliced cerebral tissues (McIlwain & Gore, 1951).

It was concluded that the metabolic effects of electrical pulses on suspensions even during phosphorylation were of a different order from those brought about by pulses of the same characteristics on sliced tissues.

Mitochondrial preparations. The preparation made and handled as described by Brody & Bain (1952) was found to behave as indicated by those authors and to afford P/O ratios (atom/atom) approaching 2, while oxidizing pyruvate. Electrical pulses were without effect on respiration or phosphorylation. These experiments were, following the earlier description, carried out in media with 0·05 M potassium salts. Under these circumstances sliced cerebral tissues would already be respiring and glycolysing at their maximal rates and these would not be affected by applied pulses (Gore & McIlwain, 1952). Further experiments were therefore carried out with media progressively falling in content of potassium salts down to 0·2 mM. Electrical pulses applied to mitochondrial preparations in such media (0·2–5·0 mM-KCl) caused on the average a small change in phosphate esterification (+8%) and in oxygen uptake (+14%; mean value without pulses, $6{\cdot}31 \pm 0{\cdot}93$; average increase with pulses $0{\cdot}86 \pm 0{\cdot}55$ μmole O_2/mg. N/hr.; see the last six lines of Table 1*C*).

Table 1. *Electrical pulses and metabolism of cerebral preparations*

Preparations were from guinea pig tissues, between concentric electrodes, 10·5–11·5 mm. apart in vessels *E* (McIlwain, 1951*b*). To these were applied alternating condenser pulses of peak voltage 18–20 v and time-constant 0·4–0·5 msec., at 100/sec. Similar electrical pulses of peak potential 8–10 v gave lesser effects in all cases. Chopped tissue was prepared according to McIlwain & Buddle (1953) and suspensions to Case & McIlwain (1951). When examined histologically, the latter were found to contain very few intact cells; most nuclei were damaged but some small blood vessels were relatively intact. Mitochondrial preparations were the fraction II–R_3 of Brody & Bain (1952) from 'homogenization' of cerebral cortex in ice-cold 0·25 M sucrose. Yeast hexokinase was Slater's (1953) preparation, and cytochrome *c* and diphosphopyridine nucleotide were omitted since in agreement with Brody & Bain (1952) they were found to be without effect on respiration and phosphorylation. Adenylic acid was used in place of adenosine triphosphate and found satisfactory; fluoride was always included; other details followed Brody & Bain's description. The experiments of section *A* were in phosphate-buffered glucose saline; in *B* and *C* values from experiments with applied electrical pulses are in italics.

Preparation	Electrode	*A.* Response to electrical pulses (% of rate in their absence): Respiration	*A.* Response to electrical pulses (% of rate in their absence): Lactic acid formation
Slice, 0·35 mm. thick, particles of 2–60 mg.	Au	209±17 (5)*	288±51 (5)*
	Mo	203±17 (5)*	276±46 (5)*
Chopped tissue, particles of 10 μg.	Au	153, 150	176, 198
Suspension	Au	100, 112	90, 109
		B. Oxygen uptake (μmoles/g./hr.)	Phosphate esterified (μmoles/g./hr.)
Suspension 1	—	79, 73	272, 264
Suspension 1	Mo	*90, 88*	*282, 330*
Suspension 2	—	101, 92	292, 260
Suspension 2	Mo	*108, 128*	*312, 306*
Suspension 2 with 5×10^{-5} M 2:4-dinitrophenol	—	83, 90	14, 0
Suspension 3	—	74, 69	210, 202
Suspension 3	Mo	*82, 83*	*234, 196*
Suspension 3 with 5×10^{-5} M 2:4-dinitrophenol	—	60, 65	8, 0
		C. Oxygen uptake (μmoles/mg. N/hr.)	Phosphate esterified (μmoles/mg. N/hr.)
Mitochondrial; 37°; 50 mM-KCl	—	5·79±0·45 (5)*	20·6±2·0 (5)*
	Mo	*6·14±0·38* (5)*	*21·6±3·6* (5)*
Mitochondrial; 25°; 50 mM-KCl	—	2·24, 2·28	7·56, 9·56
	Mo	*2·24, 1·92*	*8·08, 7·96*
Mitochondrial; 37°; 5 mM-KCl	—	6·30, 6·78	24·2, 28·4
	Mo	*7·33, 6·90*	*27·4, 27·4*
Mitochondrial; 37°; 1 mM-KCl	—	7·34, 7·83	18·8, 21·8
	Mo	*9·08, 8·00*	*22·2, 21·4*
Mitochondrial; 37°; 0·2 mM-KCl	—	5·74, 5·10	22·8, 21·6
	Mo	*6·61, 6·34*	*25·4, 24·6*

* Standard deviation and number of experiments.

Control experiments; effects on individual enzymes

Because in preceding experiments, change in oxygen uptake with applied electrical pulses is not large and manometric measurement in such systems can be affected by many events other than tissue respiration, the following control observations were carried out. They are analogous to others reported previously (McIlwain, 1951*a*) in experiments with sliced tissues.

(1) Pulses were displayed with an oscilloscope throughout metabolic experiments to ensure that electrical energy was always being expended in the medium at the same rate. Any fall in rate could lead to contraction of the gas in the manometric vessel, and thus simulate oxygen absorption. This observation was all the more necessary because experiments with 'homogenates' were briefer than with slices and periods without pulses could not always be arranged before and after the period with pulses. The pulses were alternating and so lessened the possibility of electrolysis.

(2) Medium without tissue was exposed to pulses in the vessels used in respiratory experiments. No change took place. Certain electrode metals do however cause such changes (McIlwain, 1951*a*) and electrodeposited gold can also do so with substances more labile than normal media constituents. Thus oxidation of 10^{-4} M adrenaline in the phosphate saline is catalysed under the conditions of Table 1*A*, while oxidation of glucose and pyruvate is not. Cerebral preparations might produce from media constituents more labile substances and therefore after two experiments with suspensions, the reaction mixture was centrifuged and the supernatant exposed to pulses in an electrode vessel *E* (McIlwain, 1951*b*). The mean change with four such mixtures was small but could have contributed half of the 14% apparent increase in oxygen uptake observed. Pulses also might produce material affecting respiration. This is undoubtedly the case with some metals as electrodes but molybdenum, silver and electrodeposited gold were found free from such action under the present conditions. These experiments would not however control effects of substances transiently produced by tissue suspensions or by pulses.

(3) In contrast with previous experiments on slices, those with suspensions and particulate preparations expose to a much greater degree enzyme

Table 2. *Electrical pulses and creatine phosphokinase*

Reactions were followed in a mixture containing in 3 ml., creatine phosphate (1·5 mM), adenylic acid (1·8 mM), glycylglycine (0·05 M at pH 7) and extracts prepared from suspensions of guinea pig cerebral cortex, as described by Narayanaswami (1952). After and during incubation, 0·5 ml. samples were precipitated with trichloroacetic acid and creatine phosphate-P determined, as described by Narayanaswami (1952). Reactions were at 37° in vessels *G* (Ayres & McIlwain, 1953) with concentric electrodes of the metals described. Alternating condenser pulses, peak potential 18 v and time-constant 0·5 msec., were applied as indicated.

Cerebral suspension (mg./ml.)	Electrode metal	Exposure to pulses: Before adding substrate (min.)	Exposure to pulses: During reaction	Phosphocreatine (μg. P/ml. reaction mixture): Initial	Loss at: 20 min.	Loss at: 40 min.
1	Au	0	–	84	12	40
1	Au	0	+	84	3	3
2	Au	0	–	84	51	75
2	Au	0	+	84	3	6
2·5	Au	0	–	106	56*	87†
2·5	Au	5	–	106	16*	22†
2·5	Au	33	–	106	15*	26†
2·5	Mo	5	–	106	48*	84†
2·5	Mo	33	–	106	30*	32†
2·0	Ag	0	–	88	33	67
2·0	Ag	0	+	88	0	0
2·0	Ag	40	–	88	0	0
2·0	Ag	40	+	88	0	0
2·5	Ag	2	–	83	0	8
2·5	Ag	5	–	83	0	0
2·5	Mo	0	–	103	22	72
2·5	Mo	2	–	103	25	78
2·5	Mo	20	–	103	14	52

* Loss at 30 min. † Loss at 60 min.

systems and intracellular elements directly to electrodes. Earlier observations of effects of electrical currents and pulses of various types afford examples of inhibition and acceleration recognized as probably secondary to electrolysis or to metals from electrodes (see Duclaux, 1899; Oppenheimer, 1910). Effects of applied pulses were therefore examined on two enzyme systems relevant to the control of respiratory level and phosphate transfer. Table 2 shows that creatine phosphokinase was markedly inhibited by even brief exposures to pulses employed in preceding experiments, from gold or silver electrodes. Inhibition with molybdenum electrodes was much less, but still observable (Table 2). This contrasts markedly with properties of these metals as electrodes with slices, when each afforded the same considerable increase in respiration and glycolysis (Table 1).

Liberation of inorganic phosphate from adenosine triphosphate by cerebral 'homogenates' under Gore's (1951) conditions was unaffected by pulses described in Table 2, from molybdenum electrodes.

DISCUSSION AND SUMMARY

It is to be expected that some changes immediately linking applied electrical pulses and reactions in cell-containing systems, will be reproduced in cell-free systems; and also that mitochondria may exhibit certain analogous phenomena depending on permeability barriers which nevertheless need not be the basis for reaction of cell-containing tissues. Present experiments however show pulses adequate or even a little excessive for maximal stimulation of respiration in cell-containing cerebral tissues to cause with suitable electrodes little or no change in oxygen uptake in presence of cerebral suspensions and mitochondrial preparations. Concomitant phosphorylation showed similarly little change. The changes observed in some cases may be secondary to deleterious effects induced by the pulses in sensitive intracellular systems, and of these an example has been encountered in creatine phosphokinase. Inhibition of the phosphokinase was greatly dependent on the metals used for electrodes. Comparable dependence on the electrode material was not found in working with cell-containing preparations of cerebral tissues.

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Applied Electrical Pulses and the Ammonia and Acetylcholine of Isolated Cerebral Cortex Slices

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Acetylcholine changes in the brain *in vivo* seem to be associated with its electrical activity (see Feldberg, 1945*a*). In particular it has been demonstrated that *in vivo* electrical stimulation diminishes considerably the total extractable acetylcholine of brain; further that the acetylcholine returns close to the normal level before convulsions start; during their progress the level is again lowered (Richter & Crossland, 1949). Electrical stimulation, or the administration of picrotoxin, have also been found to increase the free ammonia of the brain (Richter & Dawson, 1948) and this increase occurs before the development of convulsions.

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If similar metabolic changes could be demonstrated to occur in isolated portions of the central nervous system on the application of electrical pulses, then investigations at this level could be extended to include a study of the effect of various agents on the changes. Comparative observations on normal and abnormal cerebral tissues would also be possible. This paper gives an account of experiments with cerebral cortex slices in which ammonia and acetylcholine changes following the application of electrical pulses have been studied. The techniques developed by McIlwain (1951) for the application of electrical pulses to isolated parts of the central nervous system have been employed.

EXPERIMENTAL

Slice preparation, incubation, and the application of electrical pulses. Slices were incubated in phosphate–glucose saline containing 0·127 M-NaCl, 0·0051 M-KCl, 0·00127 M-KH_2PO_4, 0·00127 M-$MgSO_4$, 0·01 M-Na_2HPO_4 (brought to pH 7·4 with HCl), 0·00275 M-$CaCl_2$ and 0·01 M glucose, saturated with O_2 at room temperature.

Cerebral cortex slices were cut parallel to the outer surface, trimmed to fit the tissue-holding electrodes and weighed, as described by McIlwain (1951). In the ammonia experiments slices weighed 80–140 mg. and in individual experiments pairs were matched to within 15 mg. (5 mg. for acetylcholine). To eliminate from consideration, where possible, variations in metabolism which might be attributable to the different depths from the surface of the cortex at which slices were cut, slices which received pulses and those which did not were matched, in the individual experiments, with respect to the order in which they were cut. The first cut from the surface of the cortex is described as an 'outer', the second as an 'inner' slice. Except where stated otherwise, all slices were fitted into the type *D* tissue-holding electrodes and these then fixed in 5·0 ml. of saline contained in type A^1 electrode manometer vessels (McIlwain, 1951).

Incubations were under O_2 at 37°. The respiratory increase on the application of electrical pulses (McIlwain, 1951) was measured. Electrical pulses were applied as alternating condenser pulses (Ayres & McIlwain, 1953) at 100/sec., time constant 0·3–0·4 msec., and peak potential 15–17 v.

Ammonia estimations. Ammonia was estimated by micro-diffusion analysis (Conway, 1947) using 0·001 or 0·0005 N-HCl. The saline and trichloroacetic acid (TCA) used for denaturing the slices were made with ammonia-free glass-distilled water. Frequent checks on the reliability and accuracy of the method were made using a standard $(NH_4)_2SO_4$ solution. For the determination of ammonia in incubated cerebral cortex slices 3 or 4 were pooled (total fresh wt. 110–150 mg.), dropped with any adhering saline into 1·2 ml. ice-cold 10% (w/v) TCA in a tared centrifuge tube, immediately brought into suspension with the use of a close-fitting glass pestle, and the tube was re-weighed. Tubes were centrifuged at 0° in a high-speed refrigerated centrifuge and the supernatants retained for ammonia analysis. To avoid hydrolysis of tissue glutamine in the TCA, ammonia estimations on 1·0 ml. samples were carried out as soon as possible, and awaiting estimation the solutions were maintained at −15° (see Richter & Dawson, 1948).

Acetylcholine assays. Acetylcholine was assayed routinely by observing the contractions of frog rectus abdominis muscle, following essentially the procedure employed by Quastel, Tennenbaum & Wheatley (1936) with leech muscle. A 3 ml. capacity muscle bath was used, cooled by circulating cold water. The frog-Ringer solution contained 9·0 g. NaCl, 0·42 g. KCl, 0·24 g. $CaCl_2$, 0·15 g. $NaHCO_3$ and 13·5 g. glucose made up to 1350 ml. in glass-distilled water. In the muscle bath it was continuously aerated. After allowing the muscle to relax for at least 1 hr. in non-eserinized Ringer under a tension of about 1 g., Ringer containing 0·001% eserine sulphate was added 1 hr. before acetylcholine assays were started. Eserine sulphate (0·001%) was employed for the standards, for the solutions undergoing assay and during the relaxation periods. (Some slices were incubated in media containing 0·01% eserine sulphate and the media assayed using a frog muscle where the same concentration of eserine was employed throughout. It was found, however, that this relatively high concentration of eserine greatly diminished the contractile ability of the muscle and routinely 0·001% eserine sulphate was used.) The frog muscle was weighted only by the attached lever after the initial 2 hr. relaxation period.

Assays were conducted on frog muscle usually within the range 0·05–0·10 μg. acetylcholine/2 ml., in which estimation was possible to within 0·005 μg. acetylcholine/2 ml. The error was therefore 5–10%. The rectus abdominis muscle varies greatly from one frog to another (*Rana temporaria* males were used) in its ability to respond to acetylcholine, and it was sometimes necessary to set up two or three such preparations before one was found sufficiently sensitive for the assays.

Leech dorsal muscle was sometimes employed (Quastel *et al.* 1936) and found to be more sensitive, but there was not usually such good agreement between consecutive readings as could be obtained with the frog muscle. Also relaxation of the leech muscle was difficult following each contraction (see also Richter & Crossland, 1949).

Preparation of slices for assay. Preliminary experiments in which the total extractable acetylcholine of the slices was estimated, indicated the necessity to avoid too high a concentration of TCA for the denaturation and extraction of the slice since this diminished the acetylcholine contractions of frog muscle. Perchloric acid denaturation and extraction, followed by KOH precipitation of the perchlorate, left sufficient K^+ in solution for this itself to produce a contraction of the muscle. The procedure finally accepted for the estimation of total acetylcholine in the slice was as follows. At the end of the incubation period each vessel was removed from its manometer and the electrode tissue-holder transferred into a dish of warm oxygenated phosphate–glucose saline. The slice was floated out of its holder and removed into 1 ml. of 2% (w/v) TCA in a centrifuge tube, in which it was brought into suspension with a close-fitting glass pestle, immediately by hand, and then by mechanical rotation. All manipulations from the end of incubation to the suspension of the slice were carried out as quickly as possible and occupied 30–50 sec. Following suspension the pH of the contents of each centrifuge tube was brought to 2·0–2·5, with thymol blue as external indicator. After standing 1 hr. at room temp. the tubes were spun at 0°. The supernatants were decanted off, brought to pH 4·0 (bromocresol green), and stored at −15°. Immediately before acetylcholine assay the slice extracts were brought to pH 7·0 and diluted

to a known volume. Usually the extract of a slice, 80–100 mg., was made up to 2·0 ml., and except where it was required to observe the effect of cholinesterase on the extracts, eserine sulphate was included to give a final concentration of 0·001 %. For assay a measured portion of this 2·0 ml. was diluted to a known extent, of the order 1:10, with eserinized frog-Ringer.

Preparation of media for assay. Incubation media were separated promptly from the slices and filtered into ice-cooled test tubes. They were stored at −15°. In eserinized phosphate–glucose saline, acetylcholine concentrations of 0·05, 0·07 and 0·10 μg./2 ml. applied to frog muscle, and 0·03 and 0·05 μg./2 ml. applied to leech muscle gave the same contractions, within the error of the method, as equal acetylcholine concentrations applied in eserinized frog-Ringer. It was possible, therefore, to assay for acetylcholine by directly applying the incubation media to the muscle. Eserinized frog-Ringer was employed for dilution as assay required. Except when incubation media were applied to the muscle it was at all times bathed in frog-Ringer.

RESULTS

Effect of electrical pulses on the free ammonia of cerebral cortex in vitro

Preliminary experiments in which guinea pig cerebral cortex slices were incubated in saline with and without glucose indicated that some of the ammonia produced in the slice can diffuse into the surrounding medium. Thus in one experiment estimations on the media of slices incubated without glucose showed 2·3 mg. ammonia-N/100 g. wet weight brain slices at 15 min. and 7·5 mg. ammonia-N/100 g. at 30 min. If, therefore, exposure to electrical pulses *in vitro* gave an increase of ammonia in cerebral cortex slices it seemed likely that there would be some accumulation in the medium. Slices of guinea pig cerebral cortex were incubated, each in 5 ml. of phosphate–glucose saline. After 45–55 min. electrical pulses were applied for 20–25 min. in some cases. In individual experiments involving slices from the same animal, the incubation period for each slice was the same whether or not pulses were applied. A series of nine such experiments gave a mean value for the ammonia in the media of control slices, 6·0 ± 0·6 (S.E.M.) mg. N/100 g. brain slices, and where pulses were applied, 5·6 ± 0·7. The difference, 0·4 ± 0·9, was not significant ($P > 0·6$).

The ammonia content of guinea pig cerebral cortex slices has been determined after 60 min. incubation in phosphate–glucose saline. The mean of four such determinations was 0·97 ± 0·22 mg. ammonia-N/100 g. brain slices.

Effect of electrical pulses on acetylcholine of cerebral cortex in vitro

Free acetylcholine in medium. Under certain conditions electrical activity in the brain leads to a diminution in the total acetylcholine (Richter & Crossland, 1949), probably by first inducing a breakdown of the bound acetylcholine with the liberation of the free, active, readily hydrolysable form. If this sequence of events were to occur in the cerebral cortex slice, then, with eserine present to inhibit cholinesterase and with the application of electrical impulses, one would expect an increase in the acetylcholine of the incubation medium. Preliminary experiments with guinea pig cerebral cortex slices indicated that they quickly attain a stable level of total extractable acetylcholine (Table 1). It was decided to apply electrical pulses to slices which had been incubated long enough for this stable level to be attained.

Guinea pig cerebral cortex slices were incubated for 1 hr. in the absence of eserine which was added to the media at the end of the experiment. Under these conditions very small amounts of acetylcholine, sometimes < 0·25 μg./g. wet wt. brain slice, were found in the incubation media. In the absence of eserine, there was no consistent significant difference between the media of slices subjected to electrical pulses and of control slices from the same cortex.

However, when eserine sulphate (1:100000) was present to inhibit cholinesterase, a significant increase ($P < 0·01$) in the acetylcholine of the medium was observed following the application of electrical pulses (Table 2, Expt. *a*). Another measure of the significance of the increase is that in fourteen out of the seventeen experiments in this series the acetylcholine level of the medium was more than 40 % greater when pulses had been applied to the slices. The scatter of results between experiments with different animals was great, and this has been observed by others (Feldberg, 1945*b*; McLennan & Elliott, 1950). However, good agreement with respect to acetylcholine metabolism has been found between the right and left halves of the same brain in the course of these experiments (e.g. Table 2, Expt. *b*; see also Feldberg, 1945*a*). An increase in acetylcholine in the incubation medium,

Table 1. *Progress of formation of total extractable acetylcholine in incubated guinea pig cerebral cortex slices*

Incubations were carried out in the absence of eserine. Two slices were taken for each incubation and matched between separate incubations with regard to weight and the order of cutting from the cortex. Frog-rectus was used for acetylcholine assay.

Incubation period (min.)	Total extractable acetylcholine (μg./g. wet wt.)	
	Expt. 1	Expt. 2
0	< 2·0	2·9
15	4·1	5·6
30	5·9	6·1
60	4·1	6·1
120	4·2	6·0

Table 2. *Effect of electrical pulses applied to cerebral cortex* in vitro *on acetylcholine in eserinized incubation medium*

In individual experiments involving slices from the same animal, the incubation period for each slice was the same and the media of two similarly treated slices were combined for acetylcholine assay in order that at least duplicate assays might be made. For guinea pig (*a*), mean values for a series of seventeen experiments are recorded. Assays were conducted on frog rectus in the experiments with guinea pig and rabbit, and on leech in the experiment with rat.

Animal	Measurement	Total incubation period (min.)	Final period with electrical pulses (min.)	Acetylcholine in medium (μg./g. wet wt. slice)
Guinea pig (*a*)	Control	75–95	—	2·38±0·32*
Guinea pig (*a*)	Pulses applied	75–95	15–20	3·50±0·28*
Guinea pig (*a*)	Increase on application of pulses	—	—	1·12±0·32*
Guinea pig (*b*)†	—	75	—	1·02±0·03*
Rabbit	Control	100	—	2·45
Rabbit	Pulses applied	100	40	5·42
Rat	Control	100	—	4·8
Rat	Pulses applied	100	40	7·0

* S.E.M.

† 'Inner' and 'outer' slices of both hemispheres from one guinea pig were incubated floating freely and without applied impulses. Assays were on leech muscle and the mean value for five media is recorded.

Table 3. *Acetylcholine in eserinized incubation medium after varying periods of application of electrical pulses to guinea pig cerebral cortex slices*

In Expt. 1 frog-rectus was employed for acetylcholine assay and leech in Expt. 2.

Expt. no.	Total incubation period (min.)	Final period of application of electrical pulses (min.)	Acetylcholine in medium (μg./g. wet wt. of slice)
1	80	—	2·25
	80	10	2·93
	80	20	3·64
2	60	—	0·91
	75	—	1·52
	90	—	1·92
	75	15	3·70
	90	30	6·00

following the application of electrical pulses has also been observed with cerebral cortex slices of rabbit and rat (Table 2).

Experiments were carried out to obtain information on the time course of the acetylcholine increase in the medium following the application of electrical pulses. After preliminary incubation some slices received pulses for 2 min. In two cases the medium was taken for assay immediately following the application of pulses. The mean for the media of control slices was 2·04 ± 0·23 (S.E.M.) μg. acetylcholine/g. brain slice and 2·04 ± 0·23 where pulses were applied. In three experiments further incubation (10–20 min.) was allowed, following the 2 min. application of pulses, because the acetylcholine, after liberation within the slice may require time to diffuse through to the surrounding medium. The mean for the media of control slices was 3·35 ± 0·54 (S.E.M.) μg. acetylcholine/g. brain slice and 3·30 ± 0·45 where pulses were applied. The mean decrease of 0·05 ± 0·05 was not significant ($P > 0·3$).

Experiments in which slices were subjected to different periods of applied pulses indicated a progressive increase in the acetylcholine of the medium (Table 3).

Effect of electrical pulses on the total extractable acetylcholine of cerebral cortex slices. The increase in acetylcholine found in the surrounding medium on the application of electrical pulses to cerebral cortex slices could be associated with the loss of combined acetylcholine or an accelerated synthesis, or both. To examine this point the total extractable acetylcholine of slices was measured after incubation in the absence of eserine with and without the application of electrical pulses. In experiments where the acetylcholine content of the slices has been investigated eserine has not been added, in order that the free acetylcholine may be reduced to a minimum, and in order to approximate more closely to *in vivo* conditions. The results of a series of such experiments are given (Table 4, Expt. *a*) and show a significant loss ($P < 0·001$) of total acetylcholine following the application of electrical pulses to cerebral cortex slices. The loss was progressive (Table 4, Expt. *b*).

It was of interest to know to what extent the loss of total acetylcholine from a cerebral cortex slice, following the application of electrical pulses, is reversible. A slice has been allowed a period of undisturbed incubation (50 min.), following the application of electrical pulses (30 min.), before removal from the manometric vessel into trichloro-

Table 4. *Effect of electrical pulses applied to cerebral cortex* in vitro, *on total extractable acetylcholine*

Cerebral cortex slices were incubated without eserine, and frog-rectus used for acetylcholine assay. (*a*) In individual experiments involving slices from the same guinea pig the incubation period for each slice was the same and two similarly treated slices were combined for acetylcholine assay. Mean values for a series of seven experiments are given. (*b*) Experiment with rabbit.

Expt.	Total incubation period (min.)	Final period for application of pulses (min.)	Total extractable acetylcholine (μg./g. wet wt.) Control	Pulses applied	Loss
(*a*)	125–135	30	4·91 ± 0·38*	3·49 ± 0·25*	1·42 ± 0·20*
(*b*)	50	15	5·5	4·8	0·7
	65	30	5·5	3·9	1·6

* S.E.M.

Table 5. *Recovery of total acetylcholine in cerebral cortex* in vitro, *during a period of undisturbed incubation following an initial loss on the application of electrical pulses*

Guinea pig cerebral cortex slices, all 'outer' except those marked * which were 'inner', were incubated without eserine.

Expt. no.	Incubations	Total extractable acetylcholine (μg./g. wet wt.)
1	(*a*) 100 min. without pulses	5·0
	(*b*) 100 min. with pulses applied for final 30 min.	3·9
	(*c*) 150 min. without pulses	4·7
	(*d*) 150 min. without pulses final 50 min., but following 30 min. with applied pulses	4·7
2	(*e*) 95 min. without pulses	4·05
	(*f*) 95 min. with pulses applied final 30 min.	2·57
	(*g*) 125 min. without pulses	3·70*
	(*h*) 125 min. without pulses for final 30 min. but following 30 min. with applied pulses	3·50*

acetic acid (Table 5, Expt. 1). A control slice was incubated for the same period without any application of electrical pulses. Another pair of slices from the same cortex was employed to obtain assurance that the mechanism for the electrically induced loss of acetylcholine was operative. In this experiment all cortex slices used were samples of the 'outer' slices cut from the cerebral hemispheres. The agreement between the values for the slices (*c*) and (*d*) indicates a recovery of total acetylcholine in (*d*) on the cessation of electrical pulses, following a presumed initial loss indicated by the difference between (*a*) and (*b*) (Table 5, Expt. 1). A similar experiment gave essentially the same result (Table 5, Expt. 2).

Further characterization of substance contracting frog muscle

There can be little doubt that the substance estimated by its effect on frog or leech muscle, found in the incubation media or extracted from slices, with and without the application of electrical pulses, is a choline ester. In all cases where the test has been applied, hydrolysis by boiling for 30 sec. at pH 10·0, followed by restoration to pH 7·0, has abolished completely the ability of the media and the slice extracts to bring about a contraction of frog-rectus and leech muscle. When extracts of slices which had received electrical pulses and those which had acted as controls were prepared, containing no eserine, the contractions they induced in both frog-rectus and leech-dorsal muscle increased greatly after the eserinization of the muscle. The ability of these extracts to contract both frog and leech muscle disappeared on incubation at 37° for 1 hr. in frog-Ringer in the presence of cholinesterase (0·1 ml./ml.). After incubation, appropriate dilutions were made with frog-Ringer, and eserine sulphate was added to give a final concentration 0·001% before assay. Contractions were not measurably diminished after incubation with boiled cholinesterase. (I am indebted to Dr E. C. Webb for a gift of partially purified horse-serum pseudocholinesterase. 1 ml. of this enzyme hydrolysed 15 μmoles of acetylcholine/min. at 17° and pH 6·8.)

It is not possible to apply the cholinesterase test to the media since eserine must be present when the production of free acetylcholine is under investigation. The choline ester has been assayed in some cases, applying the same media or slice extracts to both leech and frog muscle. The two methods gave fair agreement (Table 6). In order to distinguish between acetylcholine and other choline esters several different pharmacological tests must be applied (Chang & Gaddum, 1933). The use of the frog-rectus and leech assays alone does not enable one to distinguish clearly between acetyl, pyruvyl and butyryl choline (Chang & Gaddum, 1933). Moreover, acetyl, pyruvyl (Matthes, 1930) and butyryl choline (Stedman, Stedman & Easson, 1932) are all hydrolysed readily by serum cholinesterase. However, acetylcholine is the main substance known to occur in animal tissues which would have the properties described, and it is therefore tentatively concluded that the substance assayed in these experiments is in fact acetylcholine.

Table 6. *Choline ester of incubation media and slices, with and without the application of electrical pulses, assayed with both frog-rectus and leech muscle*

Guinea pig cerebral cortex slices were incubated with eserine for media investigations, without eserine for slice investigations. In individual experiments the media of two similarly treated slices were combined or the slices combined for extraction.

Expt.	Total incubation period (min.)	Final application of pulses (min.)	Assay	Acetylcholine in medium (μg./g. wet wt. brain slice) Without pulses	With pulses
(*a*)	90	30	Frog	1·7	3·6
			Leech	1·1	3·8
(*b*)	90	30	Frog	2·0	4·1
			Leech	1·5	4·3
				Total extractable acetylcholine of slice (μg./g. wet wt.) Without pulses	With pulses
(*c*)	95	30	Frog	4·7	3·0
			Leech	5·4	3·8
(*d*)	85	30	Frog	2·4	1·9
			Leech	2·4	1·4

Sensitization of frog-rectus and leech muscle to acetylcholine by brain substances. It is known that there are in brain substances which will increase the sensitivity of frog-rectus (Feldberg, 1945*b*) and leech muscle (MacIntosh, 1939) to acetylcholine. The possibility has been examined that, following the application of electrical pulses, there is a decrease of sensitizing substances in cerebral cortex slices. This would give an apparent loss of total extractable acetylcholine. Acetylcholine has been removed by enzymic hydrolysis and the sensitizing ability of the extracts then tested, after the addition of a known amount of acetylcholine, by application to eserinized leech muscle. Extracts without eserine were prepared from incubated cerebral cortex slices, with and without the application of electrical impulses. Without pulses the total extractable acetylcholine, assayed on leech muscle, was 4·65 μg./g. wet wt., with pulses 2·80 μg./g. wet wt. Samples of the extracts were incubated with cholinesterase (as described in the previous section), and it was shown that the ability to contract leech muscle had disappeared. Known amounts of acetylcholine were added to other samples of the extracts, after incubation with cholinesterase, and the contractions of leech muscle given by these solutions were compared with those given by standards of the same acetylcholine concentration. No sensitization of the muscle was observed with either extract (Table 7, Expt. 1). A similar experiment was carried out with assays on frog muscle. Without pulses the total extractable acetylcholine was 4·40 μg./g. wet wt., following pulses 3·17 μg./g. wet wt. These extracts after incubation with cholinesterase to some extent sensitized the frog muscle to acetylcholine but each extract did so to the same degree (Table 7, Expt. 2).

DISCUSSION

Richter & Dawson (1948) found that the whole brain of the normal rat, killed by dropping into liquid air, contained 0·28 mg. ammonia/100 g. tissue. When rats were killed in the same way during convulsions, induced by a few seconds of electrical stimulation, this value had increased to 0·49 mg. ammonia/100 g. tissue. Under the conditions of the present investigations no evidence has been obtained of any increase in ammonia in guinea pig cerebral cortex *in vitro* following the application of electrical pulses for 20–25 min. However, the mean value of 0·97 mg. ammonia-N/100 g. tissue was obtained for guinea pig cerebral cortex slices incubated without the application of impulses. These figures may indicate that a different balance of reactions, concerned with the liberation and uptake of ammonia, exists in the isolated slice as compared with the whole brain *in vivo*.

Present studies indicate that when electrical pulses are applied to cerebral cortex slices, incubating under good nutrient conditions, there is an increase in the output of free acetylcholine into the surrounding medium. Under the same conditions there is a diminution in the total extractable acetylcholine of the slice. The acetylcholine extractable from a slice with trichloroacetic acid is, before extraction, in a different state from that which has

pharmacological activity. In brain slices (Mann, Tennenbaum & Quastel, 1938), and probably *in vivo* (Abdon & Hammarskjöld, 1944), it is in a non-diffusible form; with respect to pharmacological tests for acetylcholine it is inactive, and it is relatively immune to the action of cholinesterase. However, this combined acetylcholine readily decomposes to liberate the free active form, e.g. by shaking with chloroform, or by standing at pH 2·0–3·0 at room temperature (Mann *et al.* 1938). It seems that the application of electrical pulses to cerebral cortex *in vitro* is one means of inducing this decomposition.

Table 7. *Sensitization of leech and frog muscle to acetylcholine by extracts of guinea pig cerebral cortex after incubation* in vitro, *with and without the application of electrical pulses*

Experiment 1

Extracts were prepared from slices incubated without eserine, (*a*) 3·0 ml. from two slices (178 mg.) combined after 100 min. incubation without pulses, (*b*) 3·0 ml. from two slices (177 mg.) pooled after 100 min. incubation, with pulses applied for the final 30 min. 0·3 ml. of (*a*) and (*b*) were diluted to 3·3 ml. with frog-Ringer to include 0·001 % eserine sulphate before assay. Other 0·3 ml. samples of these extracts (*c*) and (*d*) were incubated with cholinesterase, eserine sulphate was added, and acetylcholine to a final concn. of 0·03 μg./2 ml. on dilution to 3·3 ml. with frog-Ringer. A control cholinesterase incubation (*e*) without added extract was diluted with the same additions. Leech muscle was used for acetylcholine assays.

Solution assayed	Acetylcholine (μg./2 ml.)
(*a*)	0·05
(*b*)	0·03
(*c*)	0·03
(*d*)	0·03
(*e*)	0·03

Experiment 2

Extracts were prepared from slices incubated without eserine, (*f*) 3·0 ml. from slices (225 mg.) pooled after 100 min. incubation without pulses, (*g*) 3·0 ml. from 2 slices (219 mg.) pooled after 100 min. incubation, with pulses applied for the final 30 min. 0·5 ml. of (*f*) and (*g*) were diluted to 3·3 ml. with eserinized frog-Ringer before assay. Other 0·5 ml. samples of these extracts, (*h*) and (*i*), were incubated with cholinesterase, eserine sulphate was added, and acetylcholine to a final concn. of 0·07 μg./2 ml. on dilution to 3·3 ml. with frog-Ringer. A control cholinesterase incubation (*j*) without added extract was diluted with the same additions. Frog muscle was used for acetylcholine assays.

Solution assayed	Acetylcholine (μg./2 ml.)
(*f*)	0·10
(*g*)	0·07
(*h*)	0·08
(*i*)	0·08
(*j*)	0·07

These investigations do not rule out the possibility that applied pulses also bring about an accelerated synthesis of free acetylcholine *de novo*. The precise extent to which the increase in free acetylcholine of the medium is determined by the loss of combined acetylcholine from the slice can only be assessed when both free (medium) and combined acetylcholine have been assayed for the same slices, with and without the application of electrical pulses. Present results indicate that the rate of increase in free acetylcholine in the medium, 0·056 μg./g. tissue/min. (calculated from the mean value given in Table 2) is somewhat higher than the rate of decrease of combined acetylcholine in the slice, 0·047 μg./g. tissue/min. (calculated from the mean value given in Table 4, Expt. *a*) when pulses are applied to cerebral cortex *in vitro*. It must be noted, however, that eserine was present during incubation when the acetylcholine of the medium was under investigation, but not when the combined acetylcholine of the slice was being studied.

The *in vitro* findings may be compared with the observations by Richter & Crossland (1949) of a fall in the total extractable acetylcholine of brain *in vivo*, consequent on electrical stimulation and convulsions. If electrical activity in the brain *in vivo* is associated with a release of free active acetylcholine from the combined inactive form then, since the free acetylcholine would be immediately hydrolysed under the influence of the very powerful cholinesterase of brain, the net result of such activity would be a loss in total acetylcholine. However, Richter & Crossland (1949) found the rate at which the acetylcholine of brain decreases *in vivo* on electrical stimulation, and during convulsions, to be much greater than the rates observed in the present *in vitro* studies. They found that in rats taken after 1–3 sec. of electrical stimulation there was a loss of over 50 % of the normal total acetylcholine of brain, for which they give the value 1·25 μg. acetylcholine/g. fresh brain and on the cessation of electrical stimulation this fall was followed by resynthesis of total acetylcholine of 7 μg./g. fresh brain/min. During convulsions they found a slower fall, to 50 % of the normal value in 40–50 sec., i.e. of the order 0·83 μg. acetylcholine/g. brain/min. This is some 10–20 times the rates of change observed in the separated tissue. Many variations in *in vitro* conditions however remain to be studied.

SUMMARY

1. In contrast with *in vivo* findings, no evidence has been obtained of any increase in ammonia following the application of electrical pulses to cerebral cortex slices respiring with glucose as substrate.

2. Electrical pulses applied to cerebral cortex slices respiring in eserinized phosphate–glucose saline gave a progressive increase in the incubation medium of a substance acting as free acetylcholine, able to contract frog-rectus and leech muscle. Expressed as acetylcholine, the rate of increase was of the order 0·056 μg./g. fresh tissue/min.

3. In the absence of eserine, electrical pulses brought about a progressive decrease in the combined acetylcholine of cerebral cortex slices (of the order 0·047 μg./g. fresh tissue/min.), reversible on the cessation of applied pulses.

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Rapid Changes in Creatine Phosphate Level in Cerebral Cortex Slices

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Previous studies have shown that the application of both electrical pulses and agents such as potassium chloride and 2:4-dinitrophenol to slices of cerebral cortex results in an increase in oxygen uptake and aerobic lactic acid production and usually to a fall in the level of creatine phosphate in the slice (McIlwain & Gore, 1951; Gore & McIlwain, 1952). The changes in creatine phosphate have been measured at the end of a relatively long experimental period and give no indication of the rate at which the level is lowered, nor the rate at which creatine phosphate is resynthesized after a particular treatment. Further, it has not been possible to determine the effects of different substrates or agents such as metabolic inhibitors, convulsants or depressant drugs upon these two processes.

This paper describes a method which enables these changes to be measured during the passage of electrical pulses through slices of cerebral cortex. The results obtained have been compared with some similar changes brought about by potassium chloride.

MATERIALS AND METHODS

The saline medium contained NaCl 128 mM, KCl 6·3 mM, $CaCl_2$ 2·7 mM, $MgSO_4$ 1·28 mM, glucose 10·0 mM, and aminotrishydroxymethylmethane (Tris) 25 mM. The medium was at pH 7·4. The sample of Tris was recrystallized from water before use. The saline medium contained no detectable orthophosphate when estimated as described below. In experiments in which 1·3 mM-KH_2PO_4 was present in the medium, the requisite amount of KCl was omitted in order to maintain the K^+ concentration at 6·3 mM. In experiments in which the concentration of KCl was increased, the control saline used with slices was made isosmotic with an equal molarity of added NaCl.

Technique for measurement of rapid changes in phosphates in slices of cerebral cortex

Slices of cerebral cortex of guinea pigs or rats were prepared as described by McIlwain (1951*a*). Techniques previously used for determination of changes in phosphate levels when slices were subjected to different stimuli involved too long a manipulative period to be used in the present study. An apparatus was therefore devised which permitted rapid release and fixation of a slice in a protein precipitant, after an appropriate period of stimulation. Two types of holders are shown diagrammatically in Fig. 1. Slices weighing 100–140 mg., of the cerebral cortex of guinea pigs were incubated in 3·5 ml. of Tris–glucose saline contained in 25 ml. conical flasks under an atmosphere of O_2 for 30 min. The flasks were fitted with inlet and outlet tubes closed by Bunsen valves and were shaken during incubation. The time taken between the death of the animal and placing the flasks in the thermostat was usually about 20 min. After incubation, the flask contents were transferred to a dish containing about 4–5 ml. of warm saline and the slices

were floated into the holder. The holder and slice were then incubated in saline contained in a small constant-temperature bath, the saline being stirred with a vigorous stream of O_2. Incubation was for 5 min. after which electrical pulses were applied for the required period of time, and the slice immediately released into 3–5 ml. ice-cold 10% (w/v) trichloroacetic acid (TCA) contained in a small dish cooled in ice and water. For periods of 1–3 sec. it was necessary to remove the slice and holder from the incubation bath before applying pulses in order that release of the slice into TCA could be prompt. Since similar treatment of slices in the absence of pulses did not decrease the level of the creatine phosphate (Tables 2 and 3) it was considered that this treatment did not markedly affect the metabolism of the slice. (It has also been shown by McIlwain & Rodnight (1954) that slices moistened with saline containing glucose respire at a rate similar to that obtained if the slices were floating in a large volume of saline in manometric vessels.)

For measurement of the effect of KCl, the slice, after incubation for 30 min. in saline containing an additional quantity of NaCl equivalent to the amount of KCl whose effect was being studied, was placed directly into the high-KCl saline and was removed into TCA after an appropriate period. Slices were disintegrated in TCA, in a small glass tube with a fitting pestle and the fine suspensions were centrifuged at $-5°$, at 20000 *g*. This produced a clear supernatant which was used for the separation and determination of phosphates.

Analytical methods

Orthophosphate was determined in the extracts by the method of Berenblum & Chain (1938), as modified by Long (1943). Slightly larger volumes of solution were used than were described by Long but the procedure was essentially the same.

Inorganic phosphate, adenosine di- (ADP) and tri-phosphate (ATP) and creatine phosphate were separated from the extracts by the barium-fractionation procedure of LePage (1949). Generally the 5 ml. of TCA extract was adjusted to pH 8·4 with KOH (5N and 0·1N) and 1·0 ml. of 25% (w/v) barium acetate was added to precipitate the barium-insoluble salts. This procedure gave recoveries of 94 ± 2·8% with quantities as low as 10 μg. of orthophosphate, in a total volume of 6–7 ml. This was the level of inorganic phosphate found in slices of cerebral cortex weighing 100–140 mg. wet wt. Inorganic phosphate was estimated directly in the barium-insoluble fraction and ATP was estimated as the difference between '10-min. phosphorus' and inorganic phosphorus. Creatine phosphate was determined in the barium-soluble fraction, after removal of excess of barium with sulphuric acid, by decomposition with ammonium molybdate for 30 min. at room temp. and determination of the orthophosphate formed (see Ennor & Stocken, 1948; Ernster, Zetterström & Lindberg, 1950). No attempt was made to precipitate barium creatine phosphate before analysis, since it has been adequately demonstrated (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1952*a*) that creatine phosphate is the major labile phosphate present in the fraction, separated as above, from extracts of cerebral cortex slices which had respired in a glucose-containing saline.

Lactic acid. This was estimated by the method of Barker & Summerson (1941) in samples of the saline after the slices had respired for a definite time (Table 1).

Measurement of respiration of slices of cerebral cortex, in the presence and absence of applied electrical pulses, was made in the Warburg apparatus using the ring electrode vessels type *E* (McIlwain, 1951*b*). Electrical condenser pulses were supplied at 100/sec. from the apparatus of Ayres & McIlwain (1953) using a peak voltage of 18 v with a duration of 0·4–0·5 msec. The experimental conditions were similar to those described by Heald (1953).

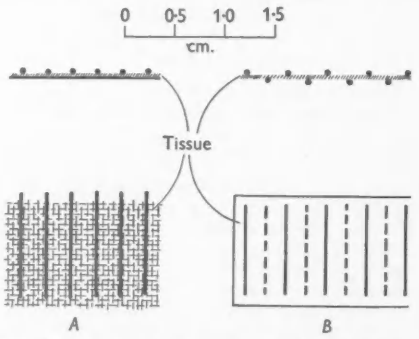

Fig. 1. A diagrammatic representation in sectional elevation and plan of the relationships between the electrodes and tissue slices. *A* represents a preliminary type of holder in which one electrode consisted of silver gauze and the other of silver wire. *B* represents a type of holder patterned after that described by Ayres & McIlwain (1953) (Fig. 2, diagrams *A–D*), in which both top and bottom electrodes were of silver wire.

RESULTS

Respiration and glycolysis in saline buffered with Tris

In Table 1 is shown the oxygen uptake and lactic acid production of slices of cerebral cortex, when metabolizing glucose in Tris–saline medium. On applying electrical pulses both respiration and glycolysis increased. The increase in lactic acid production was as great as that found in phosphate-buffered saline (McIlwain, 1951*b*), although the increase in oxygen uptake was somewhat less. Since ammonium ions at 10^{-3}M increase glycolysis in the absence of applied pulses, by some 220% (Weil-Malherbe, 1938; McIlwain & Gore, 1951), the values obtained in Table 1 indicated that ammonium salts were not present in the preparation of Tris used, in an amount sufficient to have any marked effect.

Resynthesis and maintenance of creatine phosphate

When freshly cut slices of cerebral cortex were incubated in Tris–glucose saline, creatine phosphate was resynthesized. In Table 2 (Expts. 1–3) are compared levels of creatine phosphate and inorganic phosphate both in slices which were fixed in TCA

Table 1. *Respiration and glycolysis of cerebral cortex slices in glucose–saline buffered with aminotrishydroxymethylmethane, with and without applied electrical pulses*

Each manometer vessel contained 3·5 ml. Tris–glucose saline and 70 mg. tissue slices and 0·2 ml. 5N-KOH in the centre well. Gas, O_2; temp., 37·5°. Alternating condenser pulses at 100/sec. Peak voltage: 18v. Duration of experiments 90 min. Pulses were applied during the 30th–60th min.

	O_2 consumption (μmoles/g. wet wt./hr.)			Lactic acid production (μmoles/g. wet wt. tissue)	
Animal	0–30 min.	30–60 min.	60–90 min.	With pulses	Without pulses
Rat	72	100	69	57	33
Rat	71	108	68	57·5	37
Guinea pig	54	102	55	—	—

Table 2. *Resynthesis of creatine phosphate in rat cerebral cortex slices*

Slices incubated for 30 min. in glucose–saline, buffered with aminotrishydroxymethylmethane in O_2 at 37·5°. Each value was obtained by pooling two slices of 70–80 mg. wet wt. The columns marked with an asterisk denote values obtained by transferring the slices into TCA immediately after cutting and weighing.

		Inorganic phosphate (μmoles/g. wet wt. tissue)		Creatine phosphate (μmoles/g. wet wt. tissue)	
Expt. no.	Treatment	Before* incubation	After incubation	Before* incubation	After incubation
1	Slices incubated in Tris–glucose saline for 30 min. under O_2	5·0	3·5	0·95	1·50
2		6·0	2·4	0·55	1·42
3		4·4	0·8	0·71	1·02
4	Slices incubated while held in holder *A* for 5 min., after incubation for 30 min. as above	7·6	2·4	0·95	1·40
5		7·2	1·7	0·60	1·37

Table 3. *Decrease of creatine phosphate on passage of electrical pulses through slices of cerebral cortex, in holder Type* A

Saline and pulses as in Table 1. Pulses were applied for 1 min. Slices were incubated in the holders at 37·5° in saline stirred by an oxygen stream for 5–10 min. before passing pulses. These slices had been previously incubated in small flasks as described in Methods.

	Inorganic phosphate (μmoles/g. wet wt. tissue)		Creatine phosphate (μmoles/g. wet wt. tissue)	
Expt. no.	With pulses	Without pulses	With pulses	Without pulses
1	1·70	1·80	0·66	1·20
2	2·70	2·12	0·78	1·80
3	1·78	1·60	0·68	1·20

without incubation and in slices which had been incubated in the medium containing glucose for 30 min. The levels of creatine phosphate finally reached were similar to those found in bicarbonate- and glycylglycine-buffered media (McIlwain, Buchel & Cheshire, 1951; McIlwain & Gore, 1951).

Transfer of the slices to a holder (Fig. 1), followed by further incubation of the slice in oxygenated saline, did not result in lowered levels of creatine phosphate (Table 2, Expts. 4 and 5). Since synthesis and maintenance of labile phosphates is dependent upon adequate oxygenation of the medium and hence of the tissue slice (McIlwain, 1952*a*), the results in Table 2 showed that this condition was fulfilled.

Application of electrical pulses for 1 min. to the slices when in the holders, decreased the level of creatine phosphate to that found in the slices before incubation (Table 3). From these experiments it was concluded that the saline and working conditions were adequate for the maintenance of the slices in a good metabolic condition and that the electrodes permitted response to applied electrical pulses.

Effect of electrical pulses

Role of breakdown of creatine phosphate. When slices were electrically stimulated for different periods of time, the levels of creatine phosphate and inorganic orthophosphate changed as shown in Fig. 2. This curve was obtained using a preliminary type holder *A* (Fig 1). Essentially similar results were obtained using holder *B*. The breakdown of creatine phosphate was complete in 5–6 sec. and the level was not further decreased by the passage of pulses for an additional 55 sec. The rate of breakdown of creatine phosphate in either holder *A* or *B* was 1200–1400 μmoles/g. wet tissue/hr. Concomitant with the decrease in creatine phosphate, inorganic phosphate increased. The small initial rise in the level of creatine phosphate during the first second, accompanied by a corresponding decrease in the level of inorganic phosphate was not statistically significant ($P > 0·05$).

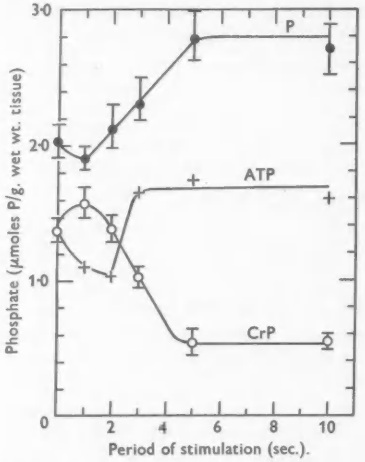

Fig. 2. Changes in creatine phosphate (CrP), inorganic phosphate (P) and adenosine triphosphate (ATP), in slices of guinea pig cerebral cortex during the passage of electrical pulses. Saline and conditions as in Methods. The horizontal lines above and below the experimental points in this and the following figures represent the limits of the S.E.M. of at least six determinations.

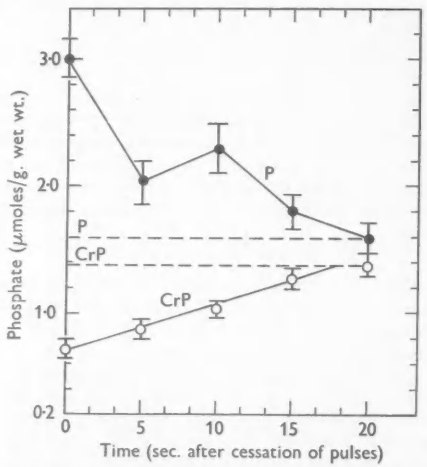

Fig. 3. The resynthesis of creatine phosphate (CrP) in slices of guinea pig cerebral cortex after the passage of impulses for 7 sec. Saline and conditions as in Methods. The dotted lines represent the levels of inorganic phosphate (P) and creatine phosphate in the slice before the passage of pulses.

Changes in adenosine triphosphate levels. In brain, as in muscle, the breakdown of creatine phosphate can be mediated by an active creatine phosphokinase (Narayanaswami, 1952). This system requires the presence of ADP, or adenylic acid, as phosphate acceptor. Previous studies had shown that in slices of cerebral cortex under good metabolic conditions, the adenosine polyphosphates were almost solely in the form of ATP with little or no ADP present (Kratzing & Narayanaswami, 1953). Since from these results it seemed that a breakdown of ATP to ADP might precede the breakdown of creatine phosphate, it was considered that a change in the level of ATP in the slices, as a result of this conversion, might be detectable during the first few seconds after passing pulses. Measurements of the acid-labile phosphate in the barium-insoluble fraction during the first 5–6 sec. of the passage of electrical pulses (Fig. 2) suggested that a decrease in ATP level did indeed precede a fall in the level of creatine phosphate.

Rate of resynthesis of creatine phosphate. Advantage was taken of the ease with which slices could be rapidly fixed after a given treatment to determine the rate of resynthesis of creatine phosphate after the passage of electrical pulses for short and long periods of time. In Fig. 3 are shown the changes in creatine phosphate and inorganic phosphate when pulses were passed through the slices for 6–7 sec. and the slices were then allowed to remain in the oxygenated saline for differing periods of time. Resynthesis of creatine phosphate was complete in about 20 sec. and was accompanied by a loss of inorganic phosphate from the slice. The rate of resynthesis was 150 μmoles/g.

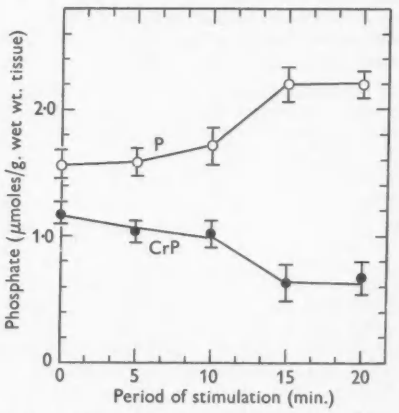

Fig. 4. The effect of the passage of electrical pulses for increasing periods of time on the ability of slices of cerebral cortex to resynthesize creatine phosphate within 20 sec. of switching off the pulses. Saline and conditions as in Methods. The ordinate represents the levels of creatine phosphate (CrP) and inorganic phosphate (P) found in the slices 20 sec. after switching off the pulses.

wet wt./hr. It was considered possible that after the passage of pulses for a long period, certain metabolites necessary for the rapid resynthesis of creatine phosphate might be depleted. Slices were treated in this way, for differing periods of time and allowed to recover for 20 sec. after cessation of pulses. The results are shown in Fig. 4, from which it is clear that continued passage of pulses decreased the ability of the slice to re-synthesize creatine phosphate.

In Fig. 5 is shown the resynthesis of creatine phosphate after passage of pulses for 20 min., both in a medium containing 1·3 mM-KH_2PO_4 and in the saline medium. The rate of resynthesis was much

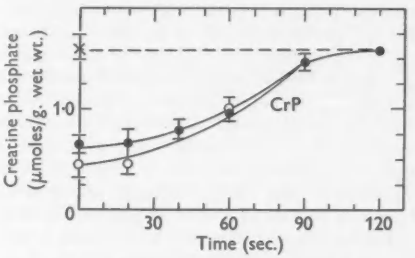

Fig. 5. The resynthesis of creatine phosphate (CrP) in slices of cerebral cortex after passing electrical pulses for 20 min. in two different salines. Saline 1, Tris–glucose saline; saline 2, Tris–glucose saline with 1·3 mM-KH_2PO_4. ○—○, Creatine phosphate resynthesis in saline 1; ●—●, creatine phosphate resynthesis in saline 2. The dotted line represents the level of creatine phosphate in slices before the passage of pulses.

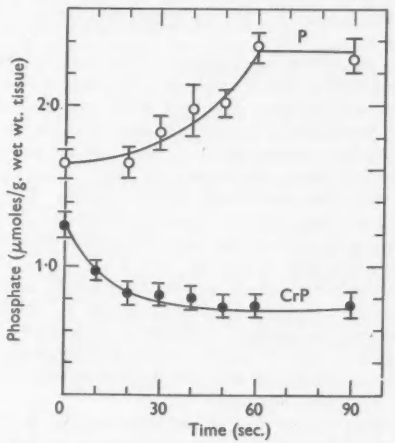

Fig. 6. The effect of 90 mM-KCl on the levels of creatine phosphate (CrP) and inorganic phosphate (P) in slices of cerebral cortex which had previously respired in Tris–glucose saline containing an additional 83·0 mM-NaCl.

slower than after applying pulses for a short period. The rate at the steepest portion, between the 80th and 90th sec., was 60 μmoles/g. wet wt./hr. Resynthesis was not affected by the presence of inorganic phosphate in the medium. The added phosphate made the values for inorganic phosphate in the slice too variable to be of use.

The effect of potassium chloride

Previous studies (McIlwain & Gore, 1951; McIlwain, 1952*a*) had shown that the increase in respiration and glycolysis when slices were treated with 0·1 M potassium chloride (Ashford & Dixon, 1935; Dickens & Greville, 1935) was accompanied by a decrease in creatine phosphate and an increase in inorganic phosphate. In this respect the effect was similar to that of electrical pulses. The similarity was greatest with 90 mM potassium chloride, at which level oxygen uptake was increased by 110% of the control and lactic acid production by 70%. When slices of cerebral cortex were incubated in a saline containing 90 mM potassium chloride for increasing periods of time the level of creatine phosphate decreased as shown in Fig. 6 falling most rapidly during the first 10 sec. at an initial rate of 120–130 μmoles/g. wet wt./hr. After 20 sec. the level of creatine phosphate had decreased by 80% of the amount of the total decrease. In contrast, during this period, the level of inorganic phosphate had not markedly increased and did not begin to do so until after the 20th sec.

DISCUSSION

The technique employed above has made possible the following of rapid changes in the levels of creatine phosphate and inorganic orthophosphate in slices of cerebral cortex when subjected to applied electrical pulses. While no attempt was made here to investigate these changes in detail, the results obtained permit a comparison of the rates of breakdown and resynthesis of creatine phosphate in intact slices, with rates of similar processes taking place either in brain *in vivo* or in brain preparations.

The effect of electrical pulses

The breakdown of creatine phosphate. On passing pulses, the level of creatine phosphate in the slices decreased at a rate of 1400 μmoles/g. wet wt./hr. This decrease did not occur immediately, but after a delay of 2–3 sec. This result differs from the *in vivo* results reported by Dawson & Richter (1950). These authors applied electrical pulses to the skulls of young rats for a period of 1 sec. or longer after which the animals were immediately dropped into liquid air. If this was done after 1 sec., brain creatine phosphate was decreased by 50% (Dawson & Richter, 1950, Fig. 1), from which a rate of breakdown

of 4600 μmoles/g. wet wt./hr. can be calculated. However, since the time taken for the transfer of the animals was about 1 sec. and the time for freezing the cortex about 4 sec., it is possible that this value is too high. Breakdown of creatine phosphate *in vivo* may therefore follow a course similar to that shown in Fig. 2.

The rate of breakdown of creatine phosphate in slices is appreciably lower than the maximum rate of 3750 μmoles/g. wet wt./hr. found by Narayanaswami (1952) for a preparation of guinea pig brain creatine phosphokinase. The rate is also lower than that of the liberation of inorganic phosphate from ATP by brain preparations, which can proceed at 1600–2100 μmoles P/g. wet wt./hr. (Gore, 1951). However, creatine phosphate is broken down at a rate greater than that required for the phosphorylation of glucose. Thus, from the data of Klein & Olsen (1947), McIlwain, Anguiano & Cheshire (1951) calculated that the maximal rate of glucose uptake by cat brain stimulated *in vivo* with electrical pulses was 370 μmoles glucose/g. wet wt./hr. during the first few seconds. This accords well with the value of 390 μmoles glucose/g. wet wt./hr. observed as the maximal rate of hexokinase activity in rat brain 'homogenates' by Long (1951). If all the glucose is phosphorylated to hexose diphosphate the maximal requirement for 'energy-rich' phosphate is about 780 μmoles/g./hr. This value is considerably exceeded by the rate of breakdown of creatine phosphate and suggests that the 'energy-rich' phosphate released in response to pulses is not used solely in the phosphorylation of glucose.

Resynthesis of creatine phosphate. In contrast to the rate of breakdown, the rate of resynthesis of creatine phosphate after electrical stimulation had ceased was 150 μmoles/g. wet wt./hr. This compares with a value of 340 μmoles/g./hr. in rat brain *in vivo* calculated from the data of Dawson & Richter (1950). The period required for complete resynthesis was 17–20 sec. This result was obtained after passage of pulses for 7 sec. If pulses were passed for a longer period (Figs. 4, 5), creatine phosphate was no longer resynthesized within 20 sec. after switching off the pulses but required up to 90 sec. The maximal rate attained was 60 μmoles/g. wet wt./hr. This slower rate of resynthesis was not altered by the presence or absence of inorganic phosphate in the medium (Fig. 5). Failure to resynthesize creatine phosphate rapidly under these conditions suggests that factors, other than glucose, oxygen and inorganic phosphate are required.

It is considered, however, that such factors are not seriously depleted during the passage of pulses for a minimal period of 7 sec. This view is supported by the results in Fig. 4 which show that even after passing impulses for 5 min., the creatine phosphate level was almost completely restored within 20 sec. of cessation of pulses. The difference in time therefore between a 7 sec. application and that required to produce a measurable effect on the rate of creatine phosphate resynthesis is very large. It is possible then to compare the rate of resynthesis shown in Fig. 3 with the rate of breakdown (Fig. 2). These rates of 150 μmoles/g. wet wt./hr. and 1400 μmoles/g. wet wt./hr. for resynthesis and degradation, respectively, clearly show that lowering of the creatine phosphate level on passage of electrical pulses is due to an increased rate of breakdown and could not be solely due to a decreased rate of synthesis. Further, it seems unlikely that the increased breakdown is due to a suppression of oxidative phosphorylation by electrical pulses. Thus, assuming a P/O ratio of 3·0 (Ochoa & Stern, 1952) in the metabolism of glucose and knowing that the rate of uptake of oxygen by slices of guinea pig cerebral cortex under the above conditions is 55 μmoles/g. wet wt./hr. (Table 1) the total requirement for 'energy-rich' phosphate to maintain a steady state in the absence of pulses could not exceed 330 μmoles/g. wet wt./hr. Since this rate is a quarter of the rate of breakdown of creatine phosphate measured above, complete cessation of oxidative phosphorylation, if it occurred, could not account for the results obtained in Fig. 2.

Relationships between inorganic phosphate, creatine phosphate and oxygen uptake. Increase or decrease in the creatine phosphate level was accompanied by a decrease or increase in inorganic orthophosphate (Figs. 2–4). The close relationships in the variation in levels of these two components suggests that the orthophosphate measured was actually derived from the phosphorus of creatine phosphate, although the pathway by which this occurs is not clear.

So far, direct measurement of oxygen uptake by slices during the shorter periods studied has not been undertaken. Previous work (see McIlwain, 1952*b*) indicates that the increase in oxygen uptake of cerebral cortex slices in response to electrical pulses is prompt and ceases abruptly within 30 sec. or less after switching off the pulses. If extra oxygen consumption ceases immediately pulses are stopped, then from the rate of synthesis of creatine phosphate (Fig. 4) and the rate of oxygen uptake of unstimulated guinea pig cerebral cortex slices of 55 μmoles O_2/g. wet wt./hr. (Table 1), the ratio creatine phosphate formed/O_2 consumed (P/O ratios) is 1·35. If extra oxygen consumption continues at the higher rate of 102 μmoles/g. wet wt./hr., the P/O ratio becomes 0·75.

These values are of an order similar to those found for oxidative phosphorylation in brain, in more artificial systems. Thus, experiments with 'homogenates' of pigeon brain in which glucose was used as phosphate acceptor yielded P/O ratios of

1·7–2·0 for the oxidation of pyruvate (Ochoa, 1941) and 1·5–3·8 with rat-brain or guinea pig brain 'homogenates' (Eiler & McEwen, 1949; Case & McIlwain, 1951). It is to be emphasized, however, that in these instances comparison is limited since the structural integrity of the slices was destroyed and the addition of fluoride, as indicated by Case & McIlwain (1951), further altered the phosphorylative capacity of the systems.

The effect of potassium salts

Although the rate of decrease of creatine phosphate was initially rapid, the time required to reach the final level (Fig. 6) was some 10 times longer than was required for a similar effect to be produced by electrical pulses. In this respect the effect of potassium salt differs from that of electrical pulses. A more marked divergence is seen in the level of inorganic phosphate which, in the case of potassium stimulation (Fig. 6), did not begin to increase until 80% of the total breakdown of creatine phosphate had occurred, suggesting that the phosphate of creatine phosphate, ultimately appearing as inorganic phosphate, was present as an intermediate during the first 20–30 sec.

SUMMARY

1. Rapid changes in the creatine phosphate and inorganic phosphate levels in slices of cerebral cortex during the passage of electrical pulses were studied in a phosphate-free medium, buffered with aminotrishydroxymethylmethane.

2. On passage of pulses, creatine phosphate was broken down at a rate of 1400 μmoles/g. wet wt. tissue/hr., after an initial lag period of 2–3 sec. Breakdown was complete in 5 sec.

3. The breakdown was preceded by a slight fall in the level of ATP, which regained its original value when creatine phosphate breakdown commenced.

4. If the pulses were switched off within 7 sec. of application, creatine phosphate was resynthesized to the original level within 20 sec. The rate of resynthesis was 150 μmoles/g. wet wt. tissue/hr. and was almost linear with time.

5. After prolonged passage of pulses creatine phosphate was resynthesized only after 90 sec. This resynthesis took place more rapidly towards the end of this period than at the beginning.

6. Treatment of slices of cerebral cortex with 0·09 M potassium chloride decreased the creatine phosphate level within 60 sec. to a level similar to that produced by electrical pulses in 5 sec.

7. The changes in level of inorganic phosphate in the slices, under the above conditions, mirrored the changes in creatine phosphate levels, with the exception of slices treated with excess of potassium chloride. In this case, the inorganic phosphate level did not rise until the creatine phosphate level had fallen by 80% of the total decrease.

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The Action of Some Ergot Derivatives, Mescaline and Dibenamine on the Metabolism of Separated Mammalian Cerebral Tissues

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The substances examined (Fig. 1) are related structurally in so far as mescaline and dibenamine are phenylalkylamines and this type of structure is discernible in lysergic acid; they act to varying degrees on the central and sympathetic nervous systems. Their effects on the metabolism of separated cerebral tissues were examined to see whether a metabolic basis for the central actions could be detected, and also in order to explore the different ways in which the separated, electrically stimulated tissue responds to added agents.

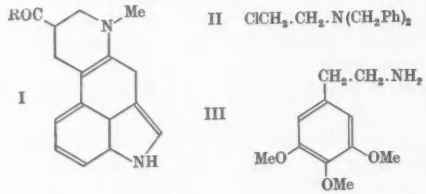

Fig. 1. Structures of substances examined. Lysergic acid diethylamide: I, R = NEt_2. Ergotoxine: a mixture of lysergic acid derivatives. Dihydroergotamine: dihydro derivative of I, R = $C_{17}H_{22}O_4N$. Dibenamine: II. Mescaline: III.

EXPERIMENTAL

Procedure

Guinea pigs were stunned by a blow on the back of the neck, bled, the brain removed, and for most experiments slices were cut as described by McIlwain (1951*a*). The dry wt. of such slices was 0·14 of their moist wt., and the moist wt. was used as basis for expressing metabolic rates. In some experiments of Table 2, tissue was weighed while fresh (dry wt./fresh wt., ratio, 0·19) and chopped into prisms 0·35 × 0·35 × 2 mm. (McIlwain & Buddle, 1953). The slices used in most experiments were either trimmed into rectangles and floated on to silver grid electrodes type *H*, which were fitted into vessels type *A*, as described by Ayres & McIlwain (1953); or cut into a number of small pieces with dissecting scissors for use in manometric electrode vessels type *E*, with ring electrodes (McIlwain, 1951*b*).

Electrical stimulation was by condenser pulses (McIlwain, 1951*a*) at a frequency of 100/sec., time constant 0·3 msec., and a peak voltage of 18 v, except in certain instances described in the text when the voltage was altered or the series of pulses interrupted (McIlwain, 1954).

Respiration was measured manometrically in Krebs–Ringer phosphate–saline (Krebs & Henseleit, 1932) in O_2, the vessels being placed in the bath at 37·5° approx. 25 min. after the death of the animal and equilibrated for 10 min. Experiments were normally of 120 min. duration, consisting of a 30 min. unstimulated period, followed by 60 min. stimulation either at one voltage throughout, or two consecutive 30 min. periods at two different voltages, the lower voltage being used first; and finally a 30 min. unstimulated period. At the end of the experiments, samples of the fluid were taken for determination of lactic acid according to the method of Barker & Summerson (1941).

Materials

Mescaline sulphate was from Roche Products, ergotoxine ethanesulphonate, B.P. (1948), from Burroughs Wellcome, lysergic acid diethylamide and dihydroergotamine methanesulphonate from Sandoz Products and dibenamine hydrochloride from L. Light and Co. Ltd. They were dissolved in saline containing neither glucose nor calcium salts, at such a concentration that addition of 0·35 ml. to the 3·15 ml. of fluid in the vessels produced the desired final concentration.

α-Oxoglutaric acid and L-glutamic acid were titrated to pH 7·2 with NaOH; lactic acid was boiled with glass-distilled water, and pyruvic acid distilled, before neutralizing. These substrates were used at a final concentration of 20 mM; glucose was at 10 mM.

RESULTS

Effects on glucose metabolism

During the present series of experiments with glucose as substrate and in the absence of applied agents the tissue respired at about 54 μmoles O_2/g. moist wt./hr., and the lactic formed during incubation for 2 hr. was about 43 μmoles/g. Applied electrical pulses increased these values to about 109 μmoles O_2/g. moist wt./hr. and 84 μmoles/g., respectively, and the agents were all found in varying concentrations to antagonize the effect of the pulses. Fig. 2 shows a typical experiment.

Greater sensitivity of stimulated metabolism. A major finding of the present experiments is that all the agents examined markedly affected the metabolism of the separated tissue in the presence of applied electrical pulses at concentrations without

effect in the absence of pulses. Figs. 2 and 3 show this effect. With most agents the respiration in the presence of pulses was sensitive to one-thirtieth of the concentration of drug needed to affect the unstimulated tissue. Action with and without pulses was particularly well differentiated in mescaline, lysergic acid diethylamide and ergotoxine. With 10^{-3}M dihydroergotamine a small inhibition of respiration occurred in the absence of pulses and increased during the experiment.

Parallel effects on respiration and glycolysis. A second generalization which can be made from the results of Fig. 3 is that the agents examined affect respiration and glycolysis in parallel. Thus mescaline at 10^{-4}M is without effect on either process, while at 3×10^{-3}M respiration is inhibited by 68% and glycolysis by 71% in the presence of applied pulses. Similar phenomena are shown by lysergic acid diethylamide between 3×10^{-6}M and 10^{-4}M, by ergotoxine between 10^{-5}M and 10^{-3}M, and by dihydroergotamine except at 3×10^{-6}M.

Dibenamine presented a somewhat different picture in that its effect increased markedly during the progress of incubation, respiration falling progressively throughout the experiments. At 10^{-5}M the initial percentage increase on stimulation (Fig. 3) was 88% but during the 60 min. stimulation period the respiration fell until it represented only 32% increase over the unstimulated rate. At 4×10^{-4}M and 6×10^{-4}M no increased respiration was seen on the passage of pulses and the respiration fell markedly until it was only 40 and 30%, respectively, of the unstimulated rate in the absence of drug.

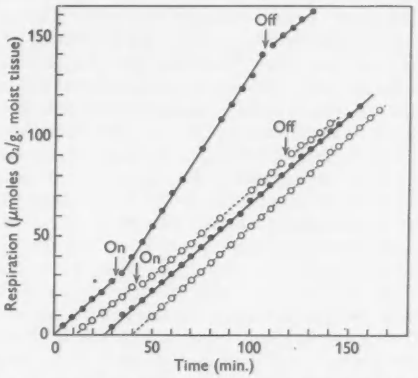

Fig. 2. Respiration of guinea pig cerebral cortex slices in the presence and absence of applied pulses (100/sec., peak potential 18 v and time-constant 0·3 msec.) with (○) mescaline (10^{-2}M) and without (●). Electrical pulses applied in two cases as indicated by the arrows. Abscissae displaced for clarity in the diagram.

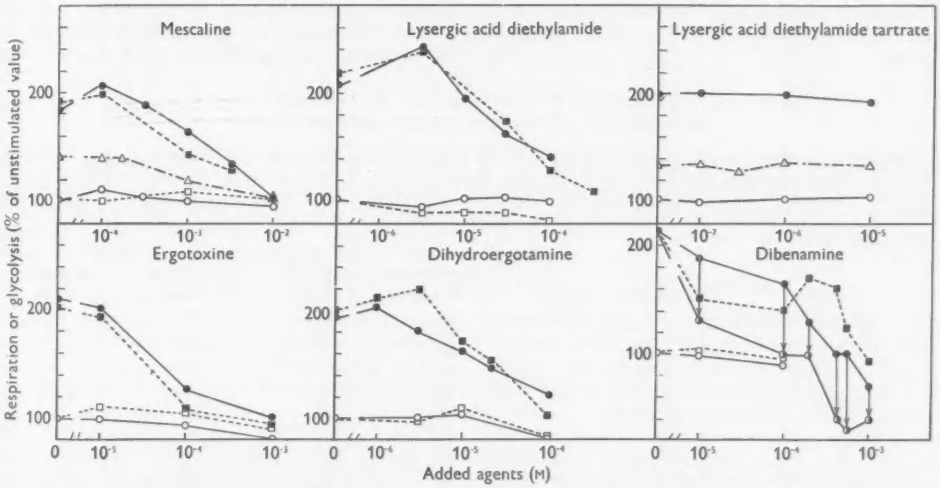

Fig. 3. Metabolism of guinea pig cerebral cortex: respiration with (●) and without (○) electrical pulses applied continuously; with interrupted pulses, △. Concomitant formation of lactic acid with (■) and without (□) pulses applied continuously. Pulses are described as continuous when applied at 100/sec., peak potential 18 v and time-constant, 0·3 msec.; and interrupted when applied in cycles of twenty such pulses followed by an interval of 2 sec. Ordinates give respiratory rates as percentages of the rate in unstimulated tissues without added agents, and the amount of lactic acid formed, again as percentage of the quantity in unstimulated controls. The arrows in the diagram with dibenamine run from points giving the result during the first 30 min. of stimulation, to those giving results during the second 30 min. Abscissae give the molar concentrations of added agents on logarithmic scales.

Lactic acid formation also showed a less regular relationship to the concentration of added inhibitor than was the case with other agents examined.

Types of electrical pulse. In the experiments described stimulation has been by pulses of exponential time–voltage relationship, applied in a continuous series. Greater sensitivity has been found to anticonvulsants (Forda & McIlwain, 1953) and to veratrine (Wollenberger, unpublished; McIlwain, unpublished), with interrupted series of pulses or with sine-wave currents. Fig. 3 shows the effects on respiration of lysergic acid diethylamide tartrate both with continuous and interrupted series of pulses. At concentrations between 10^{-5}M and 10^{-7}M, this substance was found to have little effect on the response to pulses whether continuous or interrupted. Experiments shown in the graph are with a stimulation period of 0·2 sec. every 2 sec. Other experiments (some of which are quoted in Table 2) with a stimulation-time of 0·2 sec. every 0·5 or 4 sec. similarly showed little effect. Experiments with lysergic acid diethylamide (Fig. 3) under slightly different conditions show a rise then a fall with concentrations of drug up to 10^{-5}M; this difference in response could arise due to the different conditions but does not affect the main conclusions. Mescaline also (Fig. 3) had no greater effect with interrupted pulses. Ergotoxine at concentrations of 10^{-5}M, 10^{-4}M and 10^{-3}M was found to have similar effects on respiration stimulated by sine-wave alternating currents of 50 cyc./sec. and by condenser pulses.

Sensitivity with different substrates

Table 1 shows the response of cerebral tissues with and without dihydroergotamine to applied pulses with substrates other than glucose. No response to pulses was seen when α-oxoglutaric acid or glutamic acid were substrates (McIlwain, 1951*c*; Kratzing, 1952); respiration fell throughout the experiment and no significant effect of the drug was seen. With pyruvic or lactic acids as substrates, pulses increased respiration, and dihydroergotamine almost completely prevented this increase at a concentration comparable with that acting when glucose was substrate.

Lysergic acid diethylamide was examined for its effect on stimulated metabolism of guinea pig cerebral cortex with glucose as substrate, at 2 mM as well as at the normal 10 mM, but no greater sensitivity to the drug was found. Dihydroergotamine (10^{-4}M) and ergotoxine (10^{-4}M) were without effect on the hexokinase activity of suspensions of guinea pig cerebral hemispheres, determined according to Long (1952).

Possible antagonists to ergot derivatives

Certain interactions between effects of dihydroergotamine and adrenaline, and of lysergic acid diethylamide and 5-hydroxytryptamine (serotonin) exist in the whole animal and in separated organs (Gaddum, 1953; Shaw & Woolley, 1953; and see below). Experiments to show possible interactions in separated cerebral tissues are summarized in

Table 1. *Metabolic response to electrical pulses with substrates other than glucose, in the presence of dihydroergotamine methanesulphonate*

Fragments of slices from guinea pig cerebral cortex (65–80 mg.) were in 3·5 ml. Krebs–Ringer phosphate medium in electrode vessels type *E* in O_2. Condenser pulses were applied at 100/sec., peak potential 18v and/or *10v and time-constant 0·3 msec. The drug, where present, was at 10^{-4}M in the medium before the tissue was added. All substrates were adjusted to pH 7·2 with NaOH. Results are quoted as mean values of μmoles O_2/g. moist wt./hr. with the standard error where applicable.

Substrate	No. of observations	Drug	No pulses (1st 30 min.)	With pulses (2nd and 3rd 30 min.)	No pulses (4th 30 min.)	Change with pulses (%)
α-Oxoglutaric acid	2	+	58	**52**	25	−11
	1	−	60	**48**	24	−20
	1	+	60	.	36	.
	2	−	58	.	40	.
Glutamic acid	2	+	77	**63**	40	−16
	1	−	80	**72**	52	−10
	1	+	81	.	57	.
	2	−	70	.	51	.
Pyruvic acid	2	+	51	**52**	42	+1
	2	−	59	**100**	55	+69
	3	+	54	.	54	.
	4	−	50	.	50	.
Lactic acid	8	+	70±3·8	**83**±6·0	67±5·0	+19
	12	−	67±2·3	**128**±5·1	69±2·8	+91
	6	+	65±3·1	.	.	.
	11	−	63±1·6	.	.	.
	2	+	59	**66***	58	+12
	1	−	65	**96***	70	+41

Table 2. *Absence of antagonism of dihydroergotamine by adrenaline, and of lysergic acid diethylamide by serotonin*

Slices from guinea pig cerebral cortex were used in Expt. *A*, in electrodes *H* in vessels *A* (Ayres & McIlwain, 1953) and the chopped tissue in Expt. *B* in vessels *E* (McIlwain, 1951*b*) in each case in 3·5 ml. phosphate-buffered glucose saline. The added substances were present together in the medium before the tissue was added. Condenser pulses were alternating of time-constant 0·3 msec., at intervals of 10 msec. and at the peak voltages quoted; when interrupted, the sequence was: 2 pulses applied, 50 pulses omitted, repeated throughout the 'interrupted' period. Rates are mean values from the number of experiments indicated in parentheses, and are followed by the S.E.M. where applicable.

Experiment *A*

Adrenaline (M)	Dihydroergotamine (M)	Respiratory rates: Without pulses (μmoles/g. wet wt./hr.)	Increase with pulses (%): Pulses at 8–10 v	Pulses at 18 v
0	0	57±2 (5)	81 (2)	95 (3)
10^{-4}	0	56±1·1 (7)	89 (2)	113±8 (7)
0	10^{-4}	58±1·9 (6)	27±3 (5)	21 (3)
10^{-4}	10^{-4}	59±1·9 (13)	25±13 (6)	23±6 (7)
0	2×10^{-5}	55 (2)	33 (2)	42±3 (5)
10^{-4}	2×10^{-5}	56±1·3 (6)	20 (3)	35±7·9 (4)
10^{-5}	2×10^{-5}	54 (1)	.	32 (1)
10^{-6}	2×10^{-5}	56 (1)	.	40 (1)

Experiment *B*

Serotonin (M)	Lysergic acid diethylamide (M)	Respiratory rates: Without pulses (μmoles/g. fresh wt./hr.)	Increase with pulses (%): Interrupted	Continuous
0	0	75 (4)	33 (4)	76 (4)
10^{-5}	0	75 (2)	31 (2)	72 (2)
10^{-5}	3×10^{-5}	70 (2)	14 (2)	50 (2)
0	3×10^{-5}	72 (2)	12 (2)	57 (2)
0	8×10^{-6}	74 (2)	13 (2)	67 (2)
10^{-4}	0	72 (2)	30 (2)	74 (2)
10^{-4}	3×10^{-5}	70 (2)	17 (2)	42 (2)
10^{-4}	8×10^{-6}	73 (2)	16 (2)	36 (2)

Table 2. Passage of electrical pulses in vessel *E* with either gold-plated or molybdenum-ring electrodes was found to catalyse the oxidation of adrenaline, possibly to adrenochrome. However, with electrodes *H* which were of silver wire, the comparatively slow oxidation of adrenaline which occurred in oxygenated salines was not affected by applied pulses. These electrodes were used in the experiments shown in Table 2. In the absence of adrenaline and dihydroergotamine the respiration of the separated tissue was increased by 95% over the unstimulated value. The presence of 10^{-4}M adrenaline did not alter this value significantly. Dihydroergotamine (10^{-4}M) diminished the increase in respiration due to the passage of pulses, to 21%. Addition of 10^{-4}M adrenaline did not greatly influence this value. The above results were obtained on application of pulses at a peak potential of 18 v. Similarly, no evidence for antagonism between adrenaline and dihydroergotamine was found when the voltage was lowered and the concentrations of dihydroergotamine and adrenaline were varied.

Serotonin itself had little effect on respiration of cerebral tissues or on their respiratory response to pulses, and did not decrease but tended to increase the effect of lysergic acid diethylamide.

DISCUSSION

Present findings have given several further instances in which electrically stimulated metabolism of separated cerebral tissues is more sensitive to added agents than is their metabolism as ordinarily studied. In some cases the sensitivity of the stimulated tissue approaches that necessary to explain the effects of the drugs *in vivo*, though assessment of the tissue concentration under the two conditions remains necessary. Thus dibenamine has been used as an adrenergic blocking agent at 0·5–2 m-moles/kg. (Nickerson & Goodman, 1947) and rapid intravenous injection of 2–3 m-moles/kg. can produce convulsions. Solutions of 1 mM dibenamine had appreciable effect in the present experiments. Mescaline induces hallucinations in man when

10–50 μmoles/kg. are given intravenously or orally (Guttman, 1936; Salomon, Gabrio & Thale, 1949). Present effects are seen in guinea pig tissues at 300 μM mescaline.

The ergot derivatives are active *in vivo* in man and in laboratory animals at extremely low concentrations; dihydroergotamine and components of ergotoxine affect sympathetic responses and glucose tolerance tests at intravenous doses of 10^{-3}–10^{-2} μmole/kg. and show LD_{50} doses of 0·5 and 0·02 μmole/kg., respectively (see Rothlin, 1946–7). Lysergic acid diethylamide induces hallucinations in man and changes in blood glucose, hexose monophosphate, and adrenaline at about 2×10^{-4} μmole/kg. (Stoll, 1947; Mayer-Gross, McAdam & Walker, 1952; Liddell & Weil-Malherbe, 1953). Action of the amide in the present experiments was seen at 10 μM. The amide was also examined at lower glucose concentrations in view of its effects on glucose metabolism, but no greater sensitivity observed.

Considering the effects of the present agents only on the separated electrically stimulated tissue, interesting relationships emerge which are in contrast to the effects of several other added substances. Different concentrations of the ergot alkaloids and of mescaline either inhibited both the respiratory and glycolytic responses to pulses, or affected neither. With the many different concentrations and substrates and pulse types examined, this relationship held true, whereas a different group of substances has given the opposite result (Heald, 1953). Iodoacetate and fluoride depressed glycolysis at concentrations with little action on respiration; malonate decreased stimulated respiration while increasing glycolysis. Moreover, effects of iodoacetate, fluoride and malonate on respiration changed also with change of substrate from glucose to lactate. This latter contrasting group of inhibitory agents are known to act at fairly defined points in intermediary metabolism of the unstimulated tissue. By contrast, the present agents which affect similarly the different metabolic sequelae of applied pulses, may be considered to prevent as a whole the change in level of activity normally caused by applied pulses, and thus to be acting at points more immediately connected with the tissue's response to them than the energy-yielding reactions of glucose catabolism.

SUMMARY

1. Dihydroergotamine methanesulphonate, lysergic acid diethylamide, mescaline sulphate, ergotoxine ethanesulphonate and dibenamine hydrochloride have been examined for effect on respiration and formation of lactic acid by preparations of guinea pig cerebral cortex in glucose–saline media.

2. These processes in the normal tissue were not very sensitive to the added agents, but on stimulation by electrical pulses became much more sensitive, inhibition being observed with mescaline at 10^{-3}M and with the ergot derivatives and dibenamine at 10^{-5}M, these concentrations in several cases being one-thirtieth of those which affected metabolism in the absence of pulses. Dependence of inhibition on the type of pulse applied was not demonstrated.

3. Agents within the range of concentrations in which they were effective, inhibited to similar degrees both respiration and glycolysis. No difference in the sensitivity of respiration to added agents was observed when glucose was replaced as substrate by lactic acid, pyruvic acid, α-oxoglutaric acid or glutamic acid.

4. The effect of dihydroergotamine was not prevented by the simultaneous presence of adrenaline, nor that of lysergic acid diethylamide by 5-hydroxytryptamine.

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The Metabolism of Short-chain Fatty Acids in the Sheep

3*. FORMIC, *n*-VALERIC AND SOME BRANCHED-CHAIN ACIDS

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The production of short-chain volatile fatty acids (VFA's) in the rumen of sheep is well established. Elsden (1945, 1946) used partition chromatography to confirm that acetic, propionic and butyric acids are the principal fatty acids present in rumen contents. Subsequently, other workers, using more efficient chromatographic techniques, have shown that relatively small amounts of formic, *iso*butyric, α-methylbutyric, *iso*valeric and *n*-valeric acids may also occur in the sheep rumen (cf. Annison, 1954).

Acetic, propionic and *n*-butyric acids are readily absorbed from the rumen, and many workers have shown that these acids can be metabolized in the animal body. The metabolism of these acids by various sheep tissues has been studied by Pennington (1952). The present paper concerns the metabolism in the tissues of the other acids found in the rumen. Also, although it could reasonably be assumed that these acids are absorbed from the rumen, this had not been definitely established, and experiments were undertaken to investigate this point.

Apart from their occurrence as products of fermentation, *iso*valeric and α-methylbutyric acids are probably produced during the breakdown of leucine and isoleucine respectively in animal tissues (Bloch, 1944; Coon, 1950). *iso*Butyric acid may arise similarly from valine (Kinnory & Greenberg, 1953).

A preliminary communication of this work was submitted to the Biochemical Society (Annison & Pennington, 1952).

EXPERIMENTAL

Volatile fatty acids. Racemic α-methylbutyric and *iso*valeric acids were synthesized by carbonation of the Grignard complexes of *sec.*-butyl and *iso*butyl chlorides respectively, as described by Gilman & Kirby (1932) for α-methylbutyric acid. Partial resolution of the α-methylbutyric acid was achieved by fractional crystallization of the brucine salt (Schutz & Marckwald, 1896); final preparations showed the following specific rotations: $[\alpha]_D^{22} = +5{\cdot}6 \pm 0{\cdot}01°$; $[\alpha]_D^{22} = -6{\cdot}7 \pm 0{\cdot}01°$. The reported value for the naturally occurring dextrorotatory form is $[\alpha]_D^{20} = +17{\cdot}85°$ (Marckwald, 1899). The other VFA's were 'laboratory reagent' quality (British Drug Houses Ltd.). Each acid had the calculated equivalent wt. on titration with alkali and was homogeneous when examined chromatographically by the method of James & Martin (1952). The sodium salts of the acids were employed.

Incubation of VFA's with various tissues. The general procedures described in previous papers of this series (Pennington, 1952, 1954) were employed. Residual acids were determined by steam-distillation in the Markham still, using 10 ml. samples of the medium + washings, together with 1 ml. of 10 N-H_2SO_4 and 8 g. of $MgSO_4$, $7H_2O$. When formic acid was determined 150 ml. of distillate were collected. The other acids used in the tissue incubation experiments distilled quantitatively in the first 100 ml. of distillate. The steam distillates of the media from duplicate flasks were combined after titration and retained for chromatographic analysis.

Analysis of VFA's. The gas–liquid partition chromatographic method of James & Martin (1952) was used. Improved separations of the valeric acid isomers were obtained by increasing the stearic acid content of the liquid phase (DC 550 silicone, Albright & Wilson Ltd., Oldbury, Birmingham) from 10 to 15% (w/w). The operating temperature was 135° using ethylene glycol monoethyl ether (ethyl cellosolve) in the heating jacket; the flow rate was 15 ml. N_2/min.

Estimation of VFA's in blood. Blood samples (50 ml.) were laked with 150 ml. of water and deproteinized with 25 ml. of 25% (w/v) HPO_3. The filtrates were neutralized with 5 N-KOH, concentrated *in vacuo*, acidified to pH 2–3 with 10 N-H_2SO_4 and steam distilled. The distillates were retained for chromatographic analysis.

Ketone body estimation. The method of Greenberg & Lester (1944), as modified by Pennington (1952), was used.

Estimation of sulphanilamide. Free and total sulphanilamide were determined by the method of Bratton & Marshall (1939).

Manometric methods. The effect of the acids on the respiration of rumen epithelium was measured using Warburg manometer vessels gassed with oxygen and with 0·2 ml. of 5% KOH in the centre wells to absorb CO_2. The second method of Dickens & Šimer (1931) was used when measurements were made with a gas phase of 5% CO_2–95% O_2.

RESULTS

Absorption of fatty acids from the rumen

The absorption of the VFA's from the rumen was studied *in vivo* with an ewe anaesthetized with Nembutal. The oesophagus and reticulo-omasal

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orifice were closed by ligatures and the carotid artery and posterior rumen vein exposed for removal of blood samples. The rumen was now emptied and washed out with warm water. A solution (2945 ml.) containing approximately equimolecular amounts of the sodium salts of *iso*-butyric, *n*-butyric, *iso*valeric, α-methylbutyric and *n*-valeric acids (about 0·02M with respect to each acid), potassium dihydrogen phosphate (0·016M), potassium chloride (0·011M) and sodium bicarbonate (0·02M) was warmed to 37°, saturated with carbon dioxide and introduced into the rumen through a permanent fistula. *n*-Butyric acid was included for comparison. Since it is not possible to remove the last traces of VFA from the rumen by washing out with water, a sample of the VFA mixture was removed for pH measurement and VFA analysis immediately after it had been placed in the rumen. Blood samples for ketone body and VFA estimations were taken immediately before the VFA solution was put into the rumen. Samples of blood and rumen fluid were removed for analysis at the times shown in Table 1. The volume of rumen fluid, after 150 min., fell to 2650 ml. (corrected for the fluid removed in sampling) and the pH rose from 6·6 to 7·3.

In a second experiment, under similar conditions, acetate and propionate were included in the solution (3 l.) introduced into the rumen, which again was 0·02M (approx.) with respect to each acid. The changes in the VFA content of the rumen were not followed, but blood samples were analysed as before (Table 1). The final volume of the rumen fluid, the pH of which rose from 6·5 to 7·5, was not recorded.

It will be seen that *iso*butyric, *n*-valeric and α-methylbutyric acids are all absorbed under these conditions. The relative amounts of all the acids appearing in the blood during its passage through the rumen wall may be deduced by subtracting the concentrations in the arterial blood from those in the blood draining the rumen. Values of the same order and comparable to that for *n*-butyric acid are obtained for all four of the acids. There was only a moderate rise in blood ketone bodies. The relatively large amounts of acetic acid appearing in the rumen blood when this acid was originally present in the rumen in the same concentration as the other acids (Expt. 2) is striking.

Table 1. *Changes in the concentrations of volatile fatty acids in the rumen and blood and of ketone bodies in the blood after introduction of a solution of fatty acids into the rumen*

Experiment 1

	Liquid in rumen			Blood from ruminal vein			Carotid blood	
	Initial	90 min.	150 min.	Initial	90 min.	150 min.	Initial	30 min.
Total fatty acid (m-moles/100 ml.)	10·2	8·6	7·8	0·020	0·098	0·152	.	0·035
Mol. percentage of each acid:								
Formic	0	0	0	0	9	10	.	9
Acetic	4	4	10	97	24	33	.	60
Propionic	2	1	0	3	2	1	.	3
n-Butyric	20	19	19	0	16	14	.	8
*iso*Butyric	21	21	19	0	11	10	.	5
n-Valeric	19	19	18	0	13	10	.	5
*iso*Valeric	16	18	17	0	14	11	.	5
α-Methylbutyric	18	18	17	0	12	11	.	5
Ketone bodies (μmoles/100 ml.)	.	.	.	49	100	.	41	107

Experiment 2

	Ruminal vein			Carotid blood		
	Initial	40 min.	180 min.	Initial	50 min.	180 min.
Total fatty acid (m-moles/100 ml.)	0·031	0·10	0·19	0·023	0·041	0·043
Mol. percentage of each acid:						
Formic	9	3	19	14	6	7
Acetic	90	51	44	84	64	86
Propionic	1	13	11	2	3	2
n-Butyric	0	10	5	0	10	2
*iso*Butyric	0	5	6	0	7	1
n-Valeric	0	7	5	0	2	0
*iso*Valeric	0	5	5	0	5	1
α-Methylbutyric	0	6	5	0	3	1
Ketone bodies (μmoles/100 ml.)	30	65	98	38	71	77

Table 2. *Loss of volatile fatty acid and production of ketone bodies when fatty acids incubated with sheep rumen epithelium*

Tissue incubated for 3 hr. at 39·5°. The flasks contained Ringer–phosphate (Krebs & Henseleit, 1932), or Ringer–phosphate–bicarbonate (Krebs & Henseleit, 1932, modified by Pennington, 1954), together with fatty acid as sodium salt (total vol. = 10 ml.). The flasks containing Ringer–phosphate were gassed with O_2 and those containing Ringer–phosphate–bicarbonate gassed with 80% O_2–20% CO_2. Dry wt. of tissue per flask = approx. 300 mg. The figures are calculated to 100 mg. dry wt. The ketone-body values are derived by subtraction of the corresponding control figures, shown in the table.

		Expt. 1		Expt. 2	
Substrate (100 μmoles)	CO_2	Fatty acid lost (μmoles)	Ketone bodies produced (μmoles)	Fatty acid lost (μmoles)	Ketone bodies produced (μmoles)
*iso*Butyric	–	24·2	−2·3	23·7	−2·2
	+	23·6	−2·4	23·6	−1·7
n-Valeric	–	20·2	5·8	18·3	5·3
	+	27·1	6·0	24·8	5·3
*iso*Valeric	–	1·7	−1·5	0·4	−1·0
	+	5·0	2·9	4·7	5·7
α-Methylbutyric	–	12·2	−1·7	11·9	−1·8
	+	12·5	−2·1	10·8	−1·5
None	–	−1·0*	3·1	−1·9*	3·4
	–	.	.	−1·4*	3·0

* Production of acid.

The apparent presence of formic acid in the sheep's blood was an unexpected feature of these results. In the present paper formic acid is identified by the appearance of a band in the appropriate position on the liquid–gas chromatogram and the identification is, therefore, only tentative. Subsequent work by one of us (Annison, to be published) has established more conclusively the identity of this band and has indicated that under normal conditions 10–30% of the VFA in sheep blood is formate.

Utilization of acids by rumen epithelium

In the first paper of this series (Pennington, 1952) it was shown that *in vitro* the epithelial tissue of the rumen metabolizes acetic, propionic and *n*-butyric acids. Of special interest was the observation that a large proportion of *n*-butyric acid was converted into ketone bodies by this tissue. Table 2 shows the results of similar experiments with the acids under study (except formic acid, which is considered in a later section). Each acid was incubated separately with the tissue sections both in the presence and in the absence of carbon dioxide since it was previously shown that the rate of metabolism of propionate was greatly enhanced by this gas (Pennington, 1952).

Evidently all four acids were metabolized. Whereas the metabolism of *n*-valeric acid produced an increase in ketone bodies over the controls, *iso*-butyric and α-methylbutyric acids were 'antiketogenic'. *iso*Valeric acid increased ketone body formation only when carbon dioxide was present; otherwise there were less ketones formed than in the controls. In another experiment ox rumen epithelium was used, with closely similar results.

The possibility that there might be a breakdown of some of the acids by the tissues into homologues of shorter chain length was next considered. In the experiments recorded in Table 2 only the total VFA was measured after incubation and the presence of new VFA's arising from the acid originally added would remain undetected. Also, in such an event the net loss of VFA shown in Table 2 would not give a true picture of the amount of the added VFA metabolized. In further experiments the residual VFA after incubation was analysed chromatographically. The results are shown in Table 3.

The partial breakdown to lower acids of all the VFA's examined was indicated. The results indicate that *iso*butyric, *n*-valeric and α-methylbutyric acids can give rise to both acetic and propionic acids whilst *iso*valeric acid forms acetic and *iso*butyric acids. The rate of utilization of *iso*valeric acid and possibly of α-methylbutyric acid was increased by the presence of carbon dioxide.

The α-methylbutyric acid used in the experiments described above was optically inactive. In another experiment there was found to be no difference between the rates of disappearance of volatile fatty acid when the partially resolved isomers were incubated, whether at concentrations of 0·005 or 0·04 M.

The appearance of formic acid in most of the media was indicated. The amounts found were never greatly in excess of the quantity appearing in the control flask without substrate. It is therefore unlikely that formic acid was produced by the breakdown of any of the other acids.

Table 3. *Breakdown of volatile fatty acids by rumen epithelium*

Tissue incubated for 3 hr. at 39·5° with fatty acid as sodium salt. The flasks contained Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with 80% O_2–20% CO_2). Vol. of fluid = 10 ml. Dry wt. of tissue = 290–319 mg. (Expt. 1), 285–296 mg. (Expt. 2). The ketone-body values are derived by subtraction of the corresponding control figures and the figures in parentheses are the percentages of the maximum possible production of ketones from the quantities of fatty acids utilized, assuming that 2 mol. of *n*-valeric give rise to 1 mol. of ketone body and 1 mol. of *iso*valeric gives 1·5 mol. of ketone body (see Discussion).

Substrates	CO_2	Substrate lost (μmoles)	Acids produced (μmoles)					Ketone body produced (μmoles)
			Formic	Acetic	Propionic	*n*-Butyric	*iso*Butyric	
				Experiment 1				
*iso*Butyric	–	117	4·6	7·7	12·2	0·0	0·0	.
(400 μmoles)	+	118	3·0	7·5	7·5	0·0	0·0	.
n-Valeric	–	126	3·2	15·9	47·8	0·0	0·0	.
(400 μmoles)	+	117	2·8	11·2	11·2	0·0	5·6	.
*iso*Valeric	–	14·8	2·0	5·9	0·0	0·0	11·8	.
(400 μmoles)	+	34·0	3·8	11·2	0·0	0·0	3·8	.
α-Methylbutyric	–	62·2	3·4	6·8	3·4	0·0	0·0	.
(400 μmoles)	+	78·0	0·0	10·3	20·6	0·0	0·0	.
None	+		1·4	1·4	0·0	0·0	0·0	.
				Experiment 2				
*iso*Butyric	–	79·7	1·3	6·2	5·0	0·0	0·0	.
(100 μmoles)	+	76·5	1·3	4·3	1·3	0·0	0·0	.
n-Valeric	–	98·0	2·7	9·1	21·8	0·0	0·0	30·1 (62)
(100 μmoles)	+	95·7	1·3	7·9	10·6	0·0	0·0	16·3 (34)
*iso*Valeric	–	0·0	0·0	6·5	0·0	0·0	0·0	−4·2
(100 μmoles)	+	28·0	3·6	10·9	1·8	0·0	0·9	16·3 (38)
α-Methylbutyric	–	44·7	0·0	6·2	5·5	0·0	0·0	.
(100 μmoles)	+	48·2	0·0	10·0	2·7	0·0	0·0	.
None	+	.	1·1	3·4	0·1	0·2	0·0	8·6

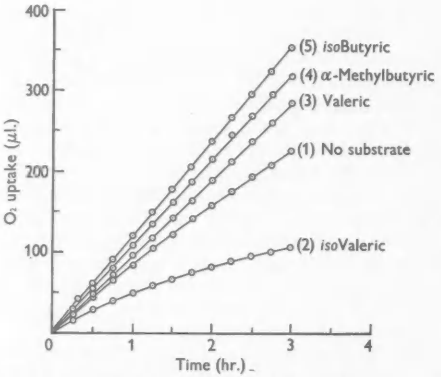

Fig. 1. Oxygen uptake of rumen epithelium in presence of fatty acids. Each flask contained 3 ml. Ringer–phosphate and 30 μmoles of the appropriate substrate. Fatty acids added as Na salts. Gas phase, oxygen. Dry wt. of tissue: 31 mg. (1, 2), 28 mg. (3, 5), 30 mg. (4).

It was thought possible that larger amounts of the breakdown products could be demonstrated by adding a suitable amine and trapping the acids as acyl derivatives, thus preventing their further breakdown. Acetylation of foreign amines by tissue slices is well known, and Schreeve (1952) demonstrated the propionylation of an aromatic amine by liver and kidney slices. Unfortunately, attempts to demonstrate acylation of sulphanilamide by rumen epithelium gave negative results. Sulphanilamide (5 mg.) was incubated with the tissue in the presence of 100 μmoles of either acetic, propionic or *n*-butyric acid. After 3 hr. there was no difference between the free and total sulphanilamide content of the medium. The rate of utilization of the fatty acids was not altered by the presence of the amine. Sulphanilamide in this concentration did not influence the oxygen uptake of the tissue.

Respiration. Fig. 1 shows the curves for oxygen uptake. All of the acids, except *iso*valeric, increased the oxygen uptake of the tissue. Owing to the relatively high endogenous respiration it was not possible to draw any conclusions from a comparison of the oxygen uptakes with the amounts of fatty acid utilized. It is not even possible to deduce minimum values for the number of oxygen atoms taken up per mole of fatty acid metabolized since oxidation of added fatty acids may stimulate the oxidation of endogenous substrates (Grafflin & Green, 1948). *iso*Valeric acid caused a marked and progressive inhibition of respiration. A similar effect had previously been observed with propionic

acid (Pennington, 1954) but only in the absence of carbon dioxide. The effect of *iso*valeric acid was therefore compared in the presence and absence of carbon dioxide, using the Dickens & Šimer (1931) second method for measurements under 5% CO_2–95% O_2 (Table 4). It is clear that like propionic acid, *iso*valeric acid only inhibits the respiration of rumen epithelium in the absence of carbon dioxide.

Utilization of acids by liver slices

Experiments similar to those with rumen epithelium recorded in Table 3 were carried out with liver slices (Table 5). *n*-Butyric acid was included for comparison. It will be seen that each of the acids was readily utilized by liver.

The effect of carbon dioxide was studied only with *iso*valeric acid; its rate of utilization was increased only slightly in the presence of a gas phase of 20% CO_2–80% O_2, when compared to the increased uptake obtained with rumen epithelium under these conditions. The metabolism of α-methylbutyric acid was associated with a small increase in ketone body production by this tissue.

The products formed were both quantitatively and qualitatively different from those obtained with rumen epithelium. Notably, there was, in general, a much smaller production of propionic acid. No *iso*butyric acid resulted from the metabolism of *iso*-valeric acid. The relatively small production of propionic acid from *n*-valeric acid was confirmed by a separate experiment (Expt. 2, Table 5).

Utilization of acids by kidney slices

Table 6 shows that all the acids were metabolized by kidney slices, the rates of utilization per unit weight of tissue being greater than that of the other two tissues, as was found previously with acetic, propionic and *n*-butyric acids. VFA production in the controls without substrate was negligible. The amount of *iso*valeric acid disappearing was about 60% greater in the presence of carbon dioxide. There was no ketone body production from any of the acids.

Utilization of formic acid by various tissues

It was not originally intended to include formic acid in these studies, since it appeared unlikely that it is normally absorbed from the rumen in appreciable quantities. It is known to be rapidly metabolized by the rumen micro-organisms (Claren, 1942). However, in view of the chromatographic evidence for the presence of this acid in both sheep blood (Table 1) and in the media in which tissues had been incubated with VFA's, experiments were carried out to determine whether it could be utilized by the tissues (Table 7). Liver was most

Table 4. *Effect of isovaleric acid upon respiration of rumen epithelium in presence and absence of carbon dioxide*

Medium: 3 ml. of Ringer–phosphate or 1·5 ml. of Ringer–bicarbonate (Krebs & Henseleit, 1932). *iso*Valeric acid, 0·01 M (as sodium salt) where added. Temp. 39·5°. Dry wt. of tissue = 25 mg. The values are computed from the readings over a 2 hr. period; Q_{O_2} = μl. O_2 consumed/mg. dry wt./hr.

Gas phase	No substrate		*iso*Valerate	
	Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
O_2	4·26	.	1·56	.
95% O_2–5% CO_2	4·12	0·60	3·90	0·64

Table 5. *Breakdown of volatile fatty acids by sheep liver slices*

Tissue incubated for 3 hr. at 39·5° with fatty acid as sodium salt. The flasks contained Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with 80% O_2–20% CO_2). Vol. of fluid = 10 ml. Dry wt. of tissue = 364–387 mg. The ketone body values are derived by subtraction of the corresponding control figures, and the figures in parentheses are the maximum possible production of ketones from the quantities of fatty acids utilized assuming that 2 mol. of *n*-valeric or α-methylbutyric acid or 1 mol. of *n*-butyric acid give rise to 1 mol. of ketone body and 1 mol. of *iso*valeric acid gives 1·5 mol. of ketone body.

Substrate (100 μmoles)	CO_2	Substrate lost (μmoles)	Acids produced (μmoles)			Ketone bodies produced (μmoles)
			Formic	Acetic	Propionic	
		Experiment 1				
*iso*Butyric	–	65·0	1·2	4·5	1·6	−5·0
n-Valeric	–	73·0	4·0	20·0	1·0	6·0 (16)
*iso*Valeric	–	59·0	0·0	7·5	0·0	35·7 (40)
	+	72·5	0·7	4·1	0·0	50·0 (41)
α-Methylbutyric	–	43·5	1·2	3·7	0·0	3·7 (18)
n-Butyric	–	62·7	0·0	6·4	0·4	34·3 (55)
None	–	.	1·7	2·1	0·0	9·9
		Experiment 2				
n-Valeric	–	38·5	3·9	14·0	0·8	.
None	–	.	1·9	4·7	0·5	.

Table 6. *Breakdown of volatile fatty acids by sheep kidney slices*

Tissue incubated for 3 hr. at 39·5° with fatty acid as sodium salt. The flasks contained Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with 80% O_2–20% CO_2). Vol. of fluid = 10 ml. Dry wt. of tissue = 122–134 mg.

Substrate (100 μmoles)	CO_2	Substrate lost (μmoles)	Acids produced (μmoles)		
			Formic	Acetic	Propionic
*iso*Butyric	–	47·2	2·4	4·8	4·8
n-Valeric	–	43·2	0·6	3·0	1·2
*iso*Valeric	–	17·8	1·3	1·3	0·0
	+	28·5	0·7	0·7	0·7
α-Methylbutyric	–	27·8	2·6	10·5	0·0

Table 7. *Utilization of formic acid by sheep and rat tissues*

Tissue incubated for 3 hr. at 39·5° in Ringer–phosphate containing sodium formate (100 μmoles). Vol. of fluid = 10 ml.; dry wt. of tissue = approx. 300 mg. The figures are calculated to 100 mg. (dry wt.) of tissue.

	Tissue	Formic acid utilized (μmoles)
Sheep	Rumen epithelium	1·5
	Liver	9·3
	Kidney	6·8
Rat	Liver	7·2
	Kidney	4·0

active in this respect. Kidney, which utilizes the other short-chain fatty acids more rapidly than liver, was less active, whilst rumen epithelium showed a negligible rate of utilization. There were no marked differences between the sheep and rat tissues in the rates of utilization. Ketone-body production by liver slices was not influenced by formate.

DISCUSSION

The metabolism of the acids studied in the experiments described above were the subject of many earlier investigations, in which interest was largely confined to ketone-body production. Jowett & Quastel (1935) found that *n*-valeric acid increased the Q_{O_2} (μl. O_2 consumed/mg. dry wt. tissue/hr.) and acetoacetate production of slices of guinea pig liver. Edson (1935) showed that both *n*- and *iso*-valeric acids were ketogenic when incubated with liver slices from well fed rats, whereas Cohen (1937) found that only the latter acid increased ketone body production. Geyer & Cunningham (1950) demonstrated the production of labelled carbon dioxide and acetoacetate by liver and kidney slices from *n*-valeric acid labelled with ^{14}C in the carboxyl group. Liver produced much more labelled acetoacetate relative to carbon dioxide than did kidney.

Early German workers (Baer & Blum, 1906; Embden, Salomon & Schmidt, 1906; Wirth, 1910), by feeding and perfusion studies, concluded that both *iso*valeric and α-methylbutyric acids were ketogenic. Demethylation was thought to be the first step in their metabolism. However, Lang & Adickes (1940), who measured ketone body production by liver slices from a wide range of branched-chain acids, found that *iso*valeric but neither α-methylbutyric nor *iso*butyric acids produced ketone bodies. Similar results were obtained by Wick (1941) when these acids were injected into rabbits. More recently, interest has centred upon the detailed mechanism of breakdown of these acids and of the amino acids from which they are derived. Bloch (1944) administered deuterium-labelled acids to rats and found that *iso*valeric but not *iso*butyric acid could provide acetyl groups for acetylation of phenylaminobutyric acid. The breakdown of *iso*-valeric acid by rat-liver slices was studied further by Zabin & Bloch (1950) and, in greater detail, by Coon (1950). By labelling the molecule in various positions Coon showed that carbon atoms 1 and 2 are split off, the 2-carbon fragments combining in pairs to give acetoacetate. This can now probably be interpreted to occur through acetyl coenzyme A formation. The remaining 3-carbon portion of the molecule apparently combines with a molecule of carbon dioxide to form acetoacetate. The stimulating effect of carbon dioxide upon *iso*valerate utilization, shown above, provides additional evidence for a pathway involving carbon dioxide fixation. The fact that this effect was less marked in liver and kidney than in rumen epithelium may be a result of a higher endogenous carbon dioxide production masking the effect. The formation of acetate from *iso*valeric acid provides more direct evidence for the occurrence of a 2-carbon product. No explanation is offered for the apparent production of *iso*butyric acid from *iso*valeric acid.

The marked inhibition of the respiration of rumen epithelium by *iso*valeric acid in an oxygen atmosphere (Fig. 1) parallels the effect of propionic acid (Pennington, 1954) and explanation along the lines of that suggested for the latter may be tentatively advanced. A complex may be formed between a moiety of the *iso*valeric acid molecule and coenzyme A or another coenzyme, which requires carbon dioxide for further metabolism. When the

concentration of carbon dioxide is low the coenzyme thus bound would be unable to function in the oxidation of endogenous substrates. It is of interest that propionic and *iso*valeric acids, both of which are metabolized more rapidly in the presence of carbon dioxide, have similar effects upon the respiration of the tissue.

Since the experiments described here were carried out, Coon and co-workers (Coon & Abrahamsen, 1952; Coon, Abrahamsen & Greene, 1952) have presented evidence, obtained from isotope studies, for a pathway of breakdown of α-methylbutyric acid by liver slices. It is postulated that β-oxidation and cleavage of the longer carbon chain occur to produce 'acetate' (which gives rise to acetoacetate) and propionate. The formation of propionate was demonstrated by isotope dilution, in which an excess of unlabelled propionate was added during the metabolism of labelled α-methylbutyric acid; about 3% of the added ^{14}C was recovered as propionate. Using rumen epithelium we were able to demonstrate the net formation of propionic acid (Table 3), but we are unable to explain why much more propionic acid is formed in the presence of carbon dioxide in Expt. 1 (Table 3).

The suppression of ketone-body formation in rumen epithelium by α-methylbutyric acid is probably connected with the formation of propionic acid, which has been shown to suppress ketogenesis from acetic acid by this tissue (Pennington & Pfander, unpublished results).

*iso*Butyric acid was studied by Atchley (1948). It was shown to be partly broken down to propionic acid by kidney 'cyclophorase' system and a mechanism involving β-oxidation to methyl malonic semialdehyde followed by decarboxylation to propionaldehyde was suggested. Recent support for breakdown via propionic acid was provided with isotope studies by Peterson, Fones & White (1952) and Kinnory & Greenberg (1953). Such a mechanism does not explain the simultaneous formation of acetic acid, strongly suggested by the data in Tables 3, 5 and 6, and possibly alternative mechanisms of breakdown exist. Valdigué & Séguélas (1946) claim to have demonstrated the formation of acetone from *iso*butyric acid, by a mechanism other than the decarboxylation of acetoacetate.

The breakdown of *n*-valeric acid into C_2 and C_3 units is presumably a result of β-oxidation. Support for this mechanism is provided by Siegel & Lorber (1951). Ketone-body formation would arise from condensation of the C_2 units in pairs.

The extensive participation of formate in intermediary metabolism has been shown by recent investigations indicating, for example, that isotope-labelled formate is incorporated into purines and pyrimidines (Lavik, 1953), acetoacetate (Plaut & Lardy, 1950), serine (Sakami, 1948; Plaut, Betheil & Lardy, 1950) and the methyl groups of choline (du Vigneaud, Verly & Wilson, 1950). Formate has been shown to be an intermediate in histidine metabolism (Soucy & Bonthillier, 1951), and the production of formate from methyl compounds incubated with liver slices was reported by Sievekitz & Greenberg (1950).

In conclusion, it seems safe to presume that any of the above-mentioned acids produced in the sheep's rumen will be utilized in the tissues and will not represent a loss of the energy of the foodstuffs to the animal.

SUMMARY

1. *iso*Butyric, *n*-valeric, *iso*valeric and α-methylbutyric acids, which are known to occur in the sheep's rumen, can be absorbed into the blood from the rumen.

2. All these acids are metabolized to some extent when incubated with rumen epithelial tissue. Small amounts of acetate and propionate were produced from *iso*butyric, *n*-valeric and α-methylbutyric acids under these conditions, whilst *iso*valeric acid gave rise to acetate and possibly *iso*butyrate. *n*-Valeric and *iso*valeric acids increased ketone-body formation.

3. The rate of metabolism of *iso*valeric acid was increased by the presence of carbon dioxide. In the absence of carbon dioxide this acid inhibited the respiration of rumen epithelium.

4. Liver and kidney slices also metabolized these acids, with breakdown to acids of shorter chain length.

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Some Esterases of the Rat

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There is much confusion in the literature on the identity of esterases and lipases. Much of this has arisen from the different conditions under which they have been examined and in particular to the variety of substrates which have been used. In most cases the preparations probably contained more than one esterase whose activities against different substrates have not been separated. For instance, although it was pointed out over 20 years ago (Cherry & Crandall, 1932) that tributyrin is not a specific substrate for pancreatic lipase, this fact has not always appeared to be appreciated. It is shown in this paper that tributyrin is an excellent substrate for both lipase and esterase.

The esterases which are discussed in this paper are not cholinesterases and do not hydrolyse choline esters. It is first necessary to try to separate the activities of the esterases (including lipases) so that the effects of alterations in the chemical nature and physical state of the substrates may be examined under strictly comparable conditions. Previous work (Aldridge, 1953*a*, *b*) has shown that the esterases of serum may be separated into two distinct types (A and B) by their sensitivity to organophosphorus inhibitors. This method has since been used for the esterases of erythrocytes (Mounter & Whittaker, 1953) and for tissue esterases (Mendel, Myers, Uyldert, Ruys & Bruyn, 1953). Using this method an initial examination of the esterases of the intestinal mucosa and pancreas of the rat soon showed that they differed markedly from those present in rat serum. A detailed examination has been made of the A-type esterases of rat serum and pancreas and the B-type esterases of intestinal mucosa. After the activities of esterases and pancreatic lipase had been clearly separated, an examination of the effect of the physical state of the substrate on the activity of both enzymes was made. This has provided a clue to the essential difference between these two enzymes. The characterization and a knowledge of the distribution of esterases should provide clues to the physiological function of this group of enzymes. The techniques described in this paper should help in approaching this problem.

MATERIALS AND METHODS

In the methods used for determining the activity of the esterases examined in this paper no claim is made that optimum conditions have been produced. No attempt has been made to determine the activity of the enzymes at their optimum pH because where several substrates are being used the pH optima may vary considerably from substrate to substrate. This has been well shown for alkaline phosphatase by Delory & King (1943) and for pancreatic lipase by Schønheyder & Volqvartz (1945*a*).

Substrates. The esters used as substrates were phenyl acetate, propionate and *n*-butyrate and triacetin and tri-*n*-butyrin from British Drug Houses Ltd.; tripropionin, trivalerin, tri-*iso*valerin, tricaproin were from Eastman Kodak Ltd. Triolein (Olive Oil B.P.) showed only a trace of free fatty acid when examined by running a 50 g. sample through an alumina column as described by Sylvester, Ainsworth & Hughes (1945). Phenyl *iso*butyrate and *iso*valerate were synthesized by the method given by Huggins & Lapides (1947). Tween 20 and Tween 80 (polyoxyethylene derivatives of sorbitan laurate and oleate respectively) were from Honeywill and Stein Ltd., London. All the phenyl esters as purchased or prepared contain free phenol which cannot be separated by distillation. The free phenol may be readily removed by passing 100 ml. of 20% (w/v) solution in dry light petroleum (B.P. below 40°) down an alumina column (type H; Peter Spence, Widnes). A further 100 ml. of light petroleum is run through and the combined solutions evaporated *in vacuo* at 50°. This procedure removes all phenol and the controls without enzyme contain only the traces of phenol liberated by the non-enzymic hydrolysis of the substrate.

Enzyme preparations. Tissue suspensions were prepared in water using a Folley & Watson type homogenizer (Folley & Watson, 1948). For many purposes acetone-dried tissue powders were used (Willstätter & Waldschmidt-Leitz, 1923). The tissue was removed and homogenized in acetone at room temperature. For rat intestinal mucosa, the small intestine of a rat was removed and washed through with running tap water. After the intestine had been cut open the mucosa was scraped off with a blunt spatula and dropped into the acetone at room temperature and then homogenized. The precipitates were centrifuged down and washed three times with acetone. The powders so obtained were dried *in vacuo* until free from acetone, ground up and finally sieved through muslin to give a fine flour-like powder which was stored at −5°. Aqueous extracts were found to be more reproducible than those prepared in buffer and to prepare extracts the powders were treated with water for 10 min. at room temperature, centrifuged and the supernatant filtered through a Whatman no. 54 filter paper. It is important when using the manometric method to add buffer to the aqueous extract before gassing with 5% CO_2 in N_2. A considerable loss of activity was observed when unbuffered extracts were used.

Pancreatic juice was obtained by Dr K. K. Cheng from rats under Nembutal anaesthesia supplemented by ether. The juice was collected through a fine polythene cannula inserted into an isolated segment of bile duct into which the pancreatic duct has been shown to drain. Secretion was stimulated by the administration of arachis oil (2 ml.) orally and by pilocarpine nitrate (160 mg./kg. body wt.) subcutaneously.

Manometric determination of enzyme activity. All determinations were carried out in the presence of 0·44% (w/v) gelatin (Bacteriological, G. T. Gurr Ltd., London) which was added to prevent inactivation of the enzymes. Buffer containing $NaHCO_3$, 0·0357 M and gelatin, 0·5% (w/v) was used except for some determinations such as the hydrolysis of the higher glycerides when buffer containing $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·040 M, and gelatin, 0·5% (w/v) was used. During the determination each flask contained 0·5 ml. of enzyme extract in water and 0·5 ml. of buffer in the side arm and 3 ml. of emulsion of substrate in buffer in the main compartment. The gaseous phase was 5% CO_2 in N_2. The final buffer concentrations during the determination of enzyme activity were therefore $NaHCO_3$, 0·0312 M; NaCl, 0·143 M; $MgCl_2$, 0·035 M; gelatin, 0·44% (w/v). The emulsions of all substrates except Tweens 20 and 80 were prepared at a concentration of approx. 20 mg./ml. in the correct buffer by homogenizing in a Folley & Watson (1948) type homogenizer at 10000 rev./min. for 2 min. The Tweens were used in 10% (v/v) concentration in buffer. During the manometric determination, readings were taken at intervals of 5 min. for the first 30 min. The output of CO_2 was in most cases linear with respect to time and the results have been obtained by calculation using the method of Aldridge, Berry & Davies (1949). At all times the rate of CO_2 evolution so obtained was proportional to enzyme concentration up to at least an output of 12 μl. of CO_2/min.

Colorimetric determination of enzyme activity using phenyl esters as substrates. A method for the estimation of enzyme activity using phenyl esters as substrates has been developed, the phenol liberated being estimated colorimetrically by the method of Gottlieb & Marsh (1946) using 4-aminoantipyrine.

Reagents: (1) Sørensen's 0·0667 M phosphate buffer, pH 7·4. (2) 0·5% (w/v) 4-Aminoantipyrine in phosphate buffer, pH 7·4; the solution is filtered and stored in the dark. The reagent is diluted 10 times with phosphate buffer before use. (3) 4% (w/v) Potassium ferricyanide in phosphate buffer, pH 7·4. The reagent is stored in the dark and diluted 10 times with phosphate buffer before use. (4) Buffered substrate. Approximately 40 mg. ester is shaken vigorously with 50 ml. of phosphate buffer, pH 7·4.

Procedure: buffered substrate mixture (3 ml.), previously warmed to 37° was added to 1·0 ml. of the enzyme preparation and the solution incubated at 37° for 15 min. The reaction was stopped by the addition of the colorimetric reagents in the following order: 4-aminoantipyrine solution (3 ml.) was added and, after mixing, 2 ml. of the potassium ferricyanide solution; the mixture was left for 5 min. and then 10 ml. of water was added to dissolve the excess substrate. The extinction at 510 mμ. was measured using the Unicam D.G. spectrophotometer. Under these conditions the rate of liberation of phenol is linear with respect to time and there is a linear relation between enzyme concentration and the phenol liberated. Occasionally with some enzyme preparations the final solution was slightly turbid; such solutions could usually be clarified by the addition of 1 ml. of dialysed iron (British Drug Houses's preparation, diluted 3 times) which removed the insoluble material but not the pigment.

Experiments with inhibitors. Solutions of inhibitors were always prepared freshly in the buffer solutions used for the determination of enzyme activity. To 0·5 ml. of tissue extract in water was added 0·5 ml. of inhibitor in buffer. After a definite incubation period the substrate was added and the enzyme activity determined. An A-type esterase is defined as one which is not inhibited (in the irreversible or phosphorylating sense, Aldridge, 1953*c*) by organophosphorus inhibitors while B-type esterases are those which are so inhibited. The inhibitors used in this paper were: di-*iso*propylphosphorofluoridate (DFP) from Allan and Hanbury Ltd., diethyl (E600) and di-*iso*propyl *p*-nitrophenylphosphates, tetraethylpyrophosphate (TEPP) from Dr H. Coates, Albright and Wilson, Ltd., *NN'*-di-*iso*propylphosphorodiamidic fluoride (mipafox) and di-*iso*propylphosphorodiamidic anhydride (DPDA) from Dr G. S. Hartley, Pest Control Ltd.

RESULTS

Esterases of serum

As previously shown (Aldridge, 1953*a*) the esterases of rat serum may be clearly differentiated into A- and B-types by their sensitivity to organophosphorus inhibitors (Table 1). After treatment with either E 600, TEPP, DFP or mipafox there is a residual activity which is due to an A-type esterase. It was previously demonstrated (Aldridge, 1953*b*) that this enzyme can hydrolyse E 600. It is therefore possible for E 600 to act as a competitive substrate in the hydrolysis of phenyl acetate. However, the concentration of substrate is much higher than that of the inhibitor and no interference with the general method for the differentiation of serum esterases into A- and B-types by means of this inhibitor is encountered.

The A-esterase of serum is sensitive to acetone and the activity of this enzyme is much reduced in acetone-dried powders of serum. This may indicate a general sensitivity to denaturation by solvents. Since in some respects the substrates may act as solvents it may explain why a linear relation between serum concentration and activity is not obtained. It was found that this could be corrected by the addition of gelatin to the buffer. A possible explanation is that the gelatin is protecting the enzyme from the denaturing action of the substrate.

In Fig. 1 is shown the substrate pattern for this enzyme against a series of phenyl esters. It has a high activity against the acetate and does not hydrolyse at a measurable rate such esters of glycerol as triacetin and tributyrin. So far as the results in this paper show, this enzyme may be termed an aromatic esterase (Mounter & Whittaker, 1953). However, it is important to remember that the aromatic esters used are more liable to non-enzymic aqueous hydrolysis than the aliphatic esters and also that aromatic rings do increase the adsorptive capacity of some compounds for protein

Table 1. *Activity of various rat-tissue preparations against esters with and without* NN′*-di-*iso*propyl-phosphorodiamidic fluoride (mipafox)*

Enzyme extracts or tissue suspensions were prepared in water and enzyme activities were determined by the manometric method. The buffer composition was $NaHCO_3$, 0·0357 M; gelatin, 0·5% (w/v). Enzyme activities are all expressed as μl. CO_2/min. obtained from 1 ml. tissue extract prepared from 2 mg. acetone-dried powder of fresh tissue. PA = Phenyl acetate, PB = phenyl butyrate, TB = tributyrin. Resistant esterase (A-type) is the esterase which remains after incubation of equal volumes of enzyme extract (in water) and 2×10^{-4} M mipafox (in buffer) for 15 min. at 37°. Total esterase was determined after a similar incubation of equal volumes of enzyme extract and buffer. For the determination of enzyme activity each flask contained 1 ml. of these mixtures or their dilution with buffer of the same concentration in the side arm and 3 ml. emulsion of substrate (approx. 20 mg./ml.) in buffer and was gassed with 5% CO_2 in N_2. The sensitive esterase (B-type) is the difference between total and resistant esterases. The same sample of rat serum was used for the experiments labelled serum (native) and serum (acetone dried); 1 g. of native serum will produce approximately 100 mg. acetone-dried powder.

Enzyme preparation	Substrate	Enzyme activity (μl. CO_2/ml./min.) Total esterase	Resistant esterase (A-type)	Sensitive esterase (B-type)
Intestinal mucosa (acetone dried)	PA	32	3	29
	PB	130	1	129
	TB	147	28	119
Pancreas (acetone dried)	PA	7	6	Nil
	PB	8	6	Nil
	TB	755	765	Nil
Pancreas (fresh tissue dispersion)	PA	—	Nil	—
	PB	—	Nil	—
	TB	—	360	—
Pancreatic juice	PA	0·39	0·26	0·13
	PB	0·65	0·55	0·10
	TB	468	455	Nil
Liver (acetone dried)	PA	17·9	2·3	15·6
	PB	17·1	Nil	17·1
	TB	3·7	Nil	3·7
Serum (native)	PA	2·56	2·18	0·38
	PB	0·79	0·09	0·70
	TB	0·098	Nil	0·098
Serum (acetone dried)	PA	5·90	3·20	2·70
	PB	5·47	0·12	5·35
	TB	0·79	Nil	0·79

(Klotz, 1946). King & Delory (1939) have also shown that phenyl phosphates are hydrolysed by alkaline phosphatase at higher rates than are simple aliphatic phosphates.

The B-type esterase of serum, like that of intestinal mucosa, is extremely sensitive to organophosphorus compounds. However, it differs from the B-type esterase of intestinal mucosa since the ratio of the rates of hydrolysis of tributyrin and phenyl *n*-butyrate is approximately 0·14 instead of 0·89.

Esterases of pancreas

The hydrolysis of tributyrin by rat serum and intestinal mucosa is prevented by treatment with 10^{-7} M mipafox. However, it was soon found that an acetone-dried powder of rat pancreas hydrolysed tributyrin rapidly but this activity was unaffected by up to 10^{-3} M mipafox (Table 1). This enzyme can therefore be classified as an A-type esterase but is clearly quite different from the A-type esterase of serum for it has little activity against phenyl esters. This preparation of pancreas does not contain any B-type esterase and this A-type enzyme appears to be the same as that present in pancreatic juice (Table 1). This A-type esterase hydrolyses higher glycerides such as triolein and is clearly pancreatic lipase.

Initially much trouble was encountered in developing a reliable method for the determination of this enzyme. Two factors are important. A solution of more consistent activity was obtained when water instead of buffer solution was used to extract the acetone-dried powder. The most important factor is however the emulsification of the substrate. The results in Table 2 indicate that for this enzyme preparation both the hydrolysis of tributyrin and triolein is increased when the substrate is emulsified. The hydrolysis of triolein is increased by magnesium while the hydrolysis of tributyrin is practically unaffected by the presence of magnesium. This is in agreement with the results and suggestion of Schønheyder & Volqvartz (1945*a*) that magnesium and calcium activate lipase by removing the higher fatty acids as they are formed as insoluble soaps. Such an activation would occur with triolein as substrate but since magnesium and calcium butyrates are soluble it should not occur with tributyrin.

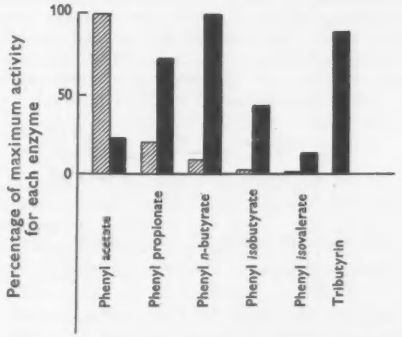

Fig. 1. Histogram of relative activities of A-type esterase of rat serum and B-type esterase of rat intestinal mucosa against phenyl esters. The preparation of A-type esterase was serum after incubation with an equal volume of 2×10^{-4} M *NN'*-di-*iso*propylphosphorodiamidic fluoride (mipafox) in buffer for 15 min. at 37°. The preparation of B-type esterase of intestinal mucosa was an extract of 20 mg. acetone-dried powder in 10 ml. of water with the addition of an equal volume of buffer after removal of the insoluble material. The buffer contained $NaHCO_3$, 0·0357 M, and gelatin, 0·5% (w/v). For the determination of enzyme activities by the manometric method each flask contained 1 ml. of these preparations or their dilutions in the side arm and 3 ml. emulsion of substrates (approx. 20 mg./ml.), and was gassed with 5% CO_2 in N_2. The activity of the B-type esterase against tributyrin is added so that Figs. 1 and 2 may be compared. ▨, A-Type esterase; ■, B-type esterase.

Table 2. *Hydrolysis of tributyrin and triolein under various conditions by extract of acetone-dried rat intestinal mucosa and pancreas*

Enzyme activities were determined by the manometric method. Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). For the determination of enzyme activity each flask contained 0·5 ml. enzyme extract in water and 0·5 ml. buffer in the side arm and 3 ml. emulsion of substrate (approx. 20 mg./ml.) and was gassed with 5% CO_2 in N_2. Enzyme activities are expressed as μl. CO_2/min. for 1 ml. of an extract of 2 mg. acetone-dried powder. For the experiments with unemulsified substrates, the ester was still in the form of large droplets at the end of the experiment.

Constituents of buffer				Enzyme activity (μl./CO_2/ml./min.)
$NaHCO_3$	NaCl	$MgCl_2$		
Pancreas extract (substrate, tributyrin)				
+	−	−	Substrate not emulsified	319
+	+	+		284
+	−	−	Substrate	986
+	+	+	emulsified	1070
Pancreas extract (substrate, triolein)				
+	−	−	Substrate not emulsified	Nil
+	+	+		Nil
+	−	−	Substrate	6·7
+	+	+	emulsified	61·2
Intestinal mucosa extract (substrate, tributyrin)				
+	−	−	Substrate not emulsified	178
+	+	+		166
+	−	−	Substrate	195
+	+	+	emulsified	174

The hydrolysis of tributyrin and triolein by an extract of acetone-dried pancreas is not inhibited by TEPP, DFP, mipafox and DPDA. However E 600 does inhibit both activities, although the inhibition curves are not the same. Previous experience with inhibitors of this type suggested that it was unlikely that only one out of a series of such compounds would inhibit the enzyme in the phosphorylating sense. Dilution experiments (Table 3) have shown that in this case E 600 is acting as a reversible inhibitor. The differing physical conditions when tributyrin and triolein are used as substrates will probably alter the effective concentration of E 600 in solution. This would account for the different inhibition curves obtained for the two substrates.

The A-type esterase of rat serum also hydrolyses E 600 as well as aromatic carboxylic esters (Aldridge, 1953*b*). The A-type esterase from rat pancreas was therefore tested for its ability to hydrolyse organophosphorus compounds. The most sensitive method to detect hydrolysis of these compounds is to incubate a high enzyme concentration with a low concentration of organophosphorus compound. Any drop in the concentration of organophosphorus compound may be detected by a drop in the inhibitory power of the solution on a test enzyme such as pseudo-cholinesterase. Table 4 gives the results of some experiments which show that no measurable hydrolysis of DFP, TEPP or mipafox occurred. Any hydrolysis of E 600 may be detected by the formation of the yellow colour of *p*-nitrophenol. No hydrolysis was observed.

Table 3. *Reversible inhibition by diethyl* p*-nitrophenylphosphate* (E 600) *of A-type esterase from rat pancreas*

Enzyme extract was prepared by extracting 20 mg. acetone-dried pancreas with 10 ml. of water. Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). *A* = a mixture of equal volumes of extract in water and $3{\cdot}9 \times 10^{-4}$ M E 600 in buffer; *B* = a mixture of equal volumes of extract and buffer. *A* and *B* were incubated for 30 min. at 37°. After dilution the enzyme activities were determined by the manometric method. For the determination each flask contained 1·0 ml. of diluted enzyme in the side arm and 3·0 ml. emulsion of tributyrin (approx. 20 mg./ml.) in buffer and was gassed with 5% CO_2 in N_2.

Dilution procedure	Enzyme activity (μl. CO_2/ml./min.)
100-Fold dilution of *A* with buffer (half-strength) containing $1{\cdot}95 \times 10^{-4}$ M E 600	Nil
100-Fold dilution of *A* with buffer (half-strength)	5·85, 5·90
100-Fold dilution of *B* with buffer (half-strength)	6·75, 6·80

Table 4. *Destruction of organophosphorus compounds by A-type esterase from rat pancreas*

Enzyme extract was prepared by extracting 20 mg. acetone-dried pancreas with 10 ml. of water. Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). Equal volumes of enzyme extract and inhibitor in buffer were mixed and incubated for 30 min. at 37°. The inhibitory power of this solution was compared against a control containing only inhibitor. Pseudo-cholinesterase was the test enzyme (0·5 ml. of four-fold diluted horse serum). For the determination of cholinesterase activity by the manometric method each flask contained 0·5 ml. inhibitory solution and 0·5 ml. diluted horse serum in the side arm and 3 ml. substrate (acetylcholine; 0·015 M final concentration) in buffer and was gassed with 5% CO_2 in N_2. The cholinesterase and inhibitory solutions were incubated in the side arm for 30 min. at 37° prior to tipping into the substrate.

Inhibitor concentration	Inhibition of cholinesterase (%)	
	Control	With pancreas extract
NN'-Di-*iso*propylphosphorodiamidic fluoride (mipafox)	64	66
Tetraethylpyrophosphate (TEPP)	86	80
Di-*iso*propylphosphorofluoridate (DFP)	83	85

Table 5. *Effects of different treatments on the ratio of the rates of tributyrin and triolein by various preparations of rat pancreas*

Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). Enzyme determinations were made using the manometric method. Each flask contained 0·5 ml. enzyme preparation in water and 0·5 ml. buffer in the side arm and 3 ml. emulsion of substrate (approx. 20 mg./ml.) and was gassed with 5% CO_2 in N_2. Nitrogen was determined by a colorimetric Nessler procedure (King, 1951). Enzyme activity (μl. CO_2/μg. N/min.) was determined using tributyrin as substrate. The substrate ratio is the ratio of the rates of hydrolysis of tributyrin and triolein.

Treatment	Enzyme activity (μl. CO_2/μg. N/min.)	Substrate ratio (tributyrin/triolein)
Fresh pancreas disrupted in water, centrifuged and filtered. Use filtrate	5·75	10·5
Water extract of freshly prepared acetone-dried pancreas	14·9	10·3
Water extract of acetone-dried pancreas (after storing for 6 months at −5°)	11·5	9·9
Precipitate water extract of acetone-dried pancreas with 19% (v/v) ethanol containing acetate buffer, pH 6·0 (cf. Cohn *et al.* 1950)	32·0	11·1
Heat water extract of acetone-dried pancreas for 10 min. at 53°	1·4	10·8

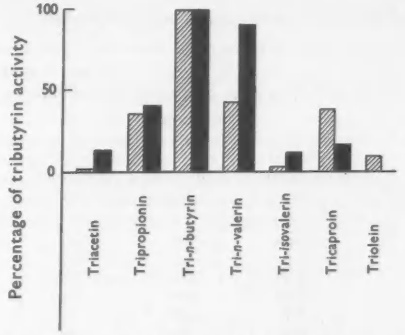

Fig. 2. Histogram of relative activities of A-type esterase of rat pancreas and B-type esterase of rat intestinal mucosa against triglycerides. The preparation of A-type esterase of pancreas was an extract of 20 mg. acetone-dried powder with 10 ml. of water with an equal volume of buffer added after removal of insoluble material. The preparation of B-type esterase of intestinal mucosa was an extract of 20 mg. acetone-dried powder in 10 ml. of water after heating for 20 min. at 56° to remove any A-type esterase present. After cooling to room temperature an equal volume of buffer was added. For the experiments with intestine the buffer contained $NaHCO_3$, 0·357 M, and gelatin, 0·5% (w/v). For the experiments with pancreas the buffer contained in addition NaCl, 0·164 M; $MgCl_2$, 0·04 M. For the determination of enzyme activities with the manometric method, each flask contained 1 ml. of these preparations or their dilutions in the side arm and 3 ml. emulsion of substrates (approx. 20 mg./ml.) and was gassed with 5% CO_2 in N_2. ▨, A-Type esterase; ■, B-type esterase.

Before the substrate pattern of the A-type enzyme of pancreas was determined it was essential to know that all the activities are mediated by the same enzyme. The results in Table 5 show that the ratio of the rates of tributyrin/triolein hydrolyses remains unaltered by a variety of treatments. It is concluded that the hydrolytic activity of this preparation against tributyrin and triolein are mediated by the same enzyme and it is assumed that the other glycerides examined are also hydrolysed by this enzyme. The relative rates of hydrolysis of these glycerides are shown in Fig. 2.

Esterases of intestinal mucosa

An acetone-dried powder of the intestinal mucosa of the rat is a very potent preparation of a B-type esterase (cf. Table 1). The equivalent of an extract of 10 μg. of such a powder will liberate 100 μg. phenol from phenyl butyrate in 15 min. under the conditions of the colorimetric technique. An advantage of the use of acetone-dried powder for this work is that although rat intestinal mucosa contains pseudo-cholinesterase (Ord & Thompson, 1950; Davison, 1953) the acetone-dried powders are almost inactive against choline esters. Its activity against butyrylcholine is only 0·5% of its activity against phenyl butyrate.

Upon treatment of this preparation with E 600 and other organophosphorus compounds its activity against phenyl butyrate is completely abolished. In Table 6 is shown its sensitivity to a variety of inhibitors of this type. It has long been known that there exist in animal tissues esterases which will hydrolyse esters such as methyl butyrate and tributyrin. These esterases are commonly called ali-esterase (Richter & Croft, 1942). Huggins & Lapides (1947) used aromatic esters and assumed that the hydrolysis obtained was due to the ali-esterase of Richter & Croft. The tributyrin hydrolysing power of rat liver is inhibited by low concentrations of E 600 (Myers & Mendel, 1953) so that it seemed probable that the B-type esterase which hydrolyses aromatic esters also will hydrolyse tributyrin. This point has been examined using initially as a test of identity the sensitivity of the activities against phenyl butyrate and tributyrin to various organophosphorus compounds. It was soon found that although its activity against phenyl butyrate was completely inhibited by organophosphorus compounds only approximately 80% of its activity against tributyrin was removed and the remaining 20% was quite resistant. Fig. 3 shows the results obtained when mipafox was used as an inhibitor. Similar results are obtained when TEPP and DFP are used but with E 600 the last 20% is inhibited by higher concentrations of inhibitor (10^{-5} M). There are present therefore in intestinal mucosa two enzymes which hydrolyse tributyrin, one of which (B-type) is inhibited by low concentrations of organophosphorus inhibitors and another which is extremely resistant (A-type).

The tributyrin hydrolysing activity of the B-type component appears to be identical with the enzyme hydrolysing phenyl butyrate. In Table 6 is shown the very close agreement of the sensitivity of both activities to treatment with five organophosphorus inhibitors. As shown in the preceding section the A-type esterase of pancreas is easily heat-denatured in unbuffered aqueous solution. While the B-type esterase of intestinal mucosa is more resistant the A-type present is also heat sensitive and by heating for 10 min. at 56° it is possible to lower the A-type esterase activity by more than 90% and only reduce the activity of the B-type esterase by 20–30%. The ratio of the rates of hydrolysis of tributyrin and phenyl butyrate (0·89) for the B-type esterase obtained after correction for the activity resistant to mipafox is the same as that obtained from a heated sample containing virtually no A-type esterase. Further heating to lower the activity of the B-type esterase itself by 70% does not alter the tributyrin to phenyl butyrate ratio. It is concluded

Table 6. *Sensitivity of B-type esterase of rat intestinal mucosa to organophosphorus compounds*

Enzyme extract (0·5 ml.; in water) was incubated with 0·5 ml. inhibitor solution in buffer for 30 min. at 37°. The residual enzyme activities were determined by the manometric method except for those marked (*), when the colorimetric method was used. Details of these methods and the buffer used are as given in the text. $pI_{50} = \log_{10}$ M concentration of inhibitor to produce 50% inhibition.

Inhibitor	Substrate: Phenyl butyrate (pI_{50})	Substrate: Tributyrin (pI_{50})	Substrate: Tween 80 (pI_{50})
Diethyl *p*-nitrophenylphosphate (E600)	8·5*	8·5	—
NN'-Di-*iso*propylphosphorodiamidic fluoride (mipafox)	7·2	7·2	7·0
Di-*iso*propylphosphorodiamidic anhydride (DPDA)	3·8*	4·1	3·9
Di-*iso*propylphosphorofluoridate (DFP)	9·0*	9·0	—
Tetraethylpyrophosphate TEPP	8·2	8·2	—
Di-*iso*propyl *p*-nitrophenylphosphate	8·6*	—	—

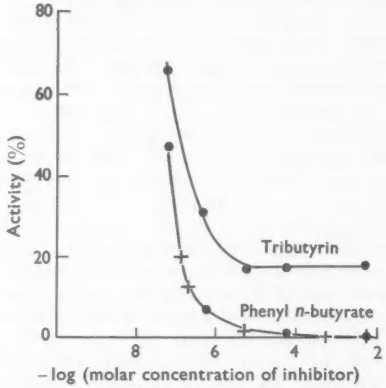

Fig. 3. Inhibition by mipafox of the hydrolysis of tributyrin and phenyl butyrate by an extract of rat intestinal mucosa. Equal volumes of an aqueous extract of the acetone-dried tissue powder and inhibitor in buffer were incubated for 30 min. at 37° prior to the addition to substrate. Enzyme activities were determined by the manometric method (●) and by the colorimetric method (+). Details of these methods are as given in the text.

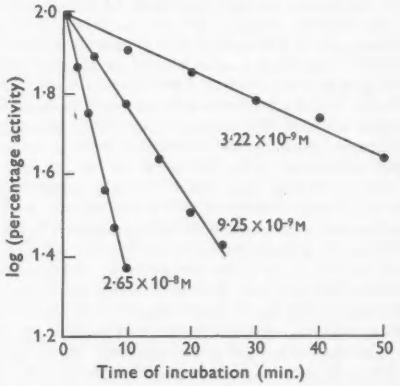

Fig. 4. Rate of loss of B-type esterase activity of rat intestinal mucosa after incubation with diethyl *p*-nitrophenylphosphate (E600). Scrapings of rat intestinal mucosa were homogenized in 0·0667 M phosphate buffer, pH 7·4, for 2 min. at room temp. with a Folley & Watson (1948) type homogenizer. Dilutions of this preparation were incubated with E600 in phosphate buffer. After various times of incubation the residual enzyme activity was determined by the colorimetric method. The addition of substrate stops the inhibitory reaction. Concentration of inhibitor during incubation shown against each curve.

therefore that these two hydrolytic activities are mediated by the same enzyme. The substrate patterns for the hydrolysis of triglycerides and phenyl esters are given in Figs. 1 and 2. The activity of this enzyme is not increased by emulsification of the substrate (Table 2). The reaction of erythrocyte cholinesterase and E600 follows the kinetics of a first-order reaction and is bimolecular (Aldridge, 1950). Similarly, the inhibition of B-type esterase of intestinal mucosa by E600 also shows the characteristics of a first-order reaction and is bimolecular (Fig. 4). The A-type esterase present in intestinal mucosa seems to be identical with the A-type esterase or lipase in pancreas. Neither enzyme is inhibited by TEPP, DFP, mipafox or DPDA. The A-type esterase of pancreas is inhibited by 10^{-5} M E600 but this is a reversible type of inhibition. The A-type esterase of intestinal mucosa is also inhibited by 10^{-5} M E600. The activity of the enzymes from both tissues against tributyrin and triolein is increased by emulsification of the substrate and both are equally sensitive to heat treatment. The ratio of the rates of hydrolysis of tributyrin and triolein for the intestinal enzyme approximates to that of the A-type esterase of pancreas. It is therefore concluded that these two enzymes are identical.

Esterases of other tissues

The B-type esterase present in the acetone-dried powder of liver, although very sensitive to organophosphorus inhibitors appears to have a substrate pattern (TB/PB = 0·22) which is different from both

Table 7. *Hydrolysis of various esters by rat brain dispersion after treatment with organophosphorus compounds*

Brain dispersions were prepared by homogenizing a rat brain in 15 ml. buffer for 2 min. at room temp. in a Folley & Watson (1948) type homogenizer. The buffer contained $NaHCO_3$, 0·0357 M; gelatin, 0·5% (w/v). Tissue dispersions (3 ml.) were incubated for 30 min. at 37° with 10^{-3} M inhibitor (0·3 ml.) in buffer. For the determination of the residual enzyme activity by the manometric method each flask contained 1 ml. of these solutions or their dilutions in buffer in the side arm and 3 ml. of substrate in buffer and was gassed with 5% CO_2 in N_2. Emulsions of the non-choline substrates (approx. 20 mg./ml.) were used. PA = Phenyl acetate, PB = phenyl butyrate, TB = tributyrin, TC = tricaproin. The choline esters were used at a final concentration of 0·015 M. AcCh = Acetylcholine, BuCh = butyrylcholine. Enzyme activities are given as μl. CO_2/min. for 1 ml. of original tissue dispersion.

Inhibitor	Substrate					
	AcCh	BuCh	PA	PB	TB	TC
	Enzyme activity (μl. CO_2/ml./min.)					
None	10·0	2·6	56·0	20·8	7·8	5·4
NN'-Di-*iso*propylphosphorodiamidic fluoride (mipafox)	0·9	Nil	21·9	10·0	3·7	3·7
Tetraethylpyrophosphate (TEPP)	Nil	Nil	9·8	2·3	1·5	2·9
Diethyl *p*-nitrophenylphosphate (E 600)	0·2	Nil	8·7	2·0	1·0	2·7

the B-type esterase of intestinal mucosa and of serum.

Recently Mendel *et al.* (1953) have stated that there is in rat brain an esterase which is less sensitive to E 600 than that in rat serum. In Table 7 is shown the results of some experiments with preparations of rat brain. This work is not yet complete but illustrates the complexity of the problem. In the first place there is a considerable activity remaining against phenyl acetate, phenyl butyrate, tributyrin and tricaproin after treatment with mipafox. The activity against phenyl butyrate cannot all be due to A-type esterase of serum and is an unusual finding. TEPP and E 600 reduce the activity against all four substrates further. These results indicate that the esterases in brain are different from those present in the other tissues previously discussed.

A comparison of the A-type esterase from pancreas with B-type esterase from intestinal mucosa

An examination of the experimental results illustrated in Fig. 2 shows that A-type esterase from pancreas and the B-type esterase from intestinal mucosa both hydrolyse the lower triglycerides but that triolein is only hydrolysed by the A-type esterase. It is clear from the evidence previously discussed that this A-type enzyme is pancreatic lipase whereas the B-type esterase is intestinal esterase. Since these two enzymes have been definitely separated, it is possible to compare their properties under identical conditions. Using tributyrin as substrate it has been shown that the activity of the B-type esterase is unaffected by emulsifying the substrate. The replacement of hydrolysed substrate is therefore not a rate-determining step. However emulsification of tributyrin increases the activity of A-type esterase three- to four-fold. These observations could be explained if it were assumed that the B-type esterase only hydrolyses the substrate in solution in the aqueous phase while the A-type esterase can adsorb on the surface of the substrate and is dependent on the surface area of the substrate available. On this basis the reason the B-type esterase does not hydrolyse triolein is not because it is incapable of hydrolysing esters of higher fatty acids but because triolein is so insoluble in water; conversely a water-soluble oleate should be hydrolysed by the B-type esterase. That this is so is shown by the fact that the B-type esterase will hydrolyse Tween 80, a water-soluble oleate. That this activity is due to B-type esterase is shown by the fact that it is inhibited by mipafox and DFP to the same extent as the hydrolysis of tributyrin and phenyl butyrate (Table 6). Tween 20 (a laurate) is also hydrolysed by B-type esterase. The ratio of the tributyrin and Tween 80 hydrolyses for B-type esterase is approximately 150 and for A-type esterase 450. The B-type esterase is therefore a little more efficient (relative to the hydrolysis of tributyrin) at the hydrolysis of the water-soluble oleate than is the A-type esterase.

The hydrolysis of choline esters by A- and B-type esterases

It has been shown by many workers that both true and pseudo-cholinesterases hydrolyse a variety of esters other than choline esters (Whittaker, 1951). It is of interest therefore to examine the esterases discussed in this paper to see if they will hydrolyse choline esters at all. The results in Table 8 show that choline esters are hardly if at all hydrolysed by these esterases. This indicates that substrates which contain a strong positive charge are probably unable to reach the enzyme active centre. This is in agreement with the view put forward by Wilson, Levine & Freiberger (1952) based on results obtained using the inhibitor prostigmine and horse-liver esterase.

Table 8. *Hydrolysis of choline esters by rat esterases*

A-Type esterase (serum) was rat serum after incubation with an equal volume 2×10^{-4}M *NN'*-di-*iso*propylphosphorodiamidic fluoride (mipafox) in buffer for 15 min. at 37° to remove B-type esterase and cholinesterases. A-Type esterase (pancreas) was an extract prepared from 80 mg. acetone-dried pancreas in 10 ml. of water after incubation with an equal volume of 2×10^{-4}M mipafox in buffer. B-Type esterase (intestine) was an extract prepared from 80 mg. acetone-dried intestinal mucosa after incubation with an equal volume of $2{\cdot}2 \times 10^{-5}$M di-*iso*propylphosphorodiamidic anhydride (DPDA) for 30 min. at 37° to remove pseudo-cholinesterase. Buffer for the experiments with serum and intestine contained $NaHCO_3$, 0·0357M, and gelatin, 0·5% (w/v). For the experiments with pancreas the buffer contained in addition NaCl, 0·164M, and $MgCl_2$, 0·04M. For the determinations of enzyme activity by the manometric method the flasks contained 1 ml. of these solutions or their dilutions and 3 ml. substrate in buffer and was gassed with 5% CO_2 in N_2. Emulsions of phenyl acetate and tributyrin (approx. 20 mg./ml.) were used and acetyl- and butyryl-choline to give a final concentration of 0·015M. Enzyme activities are given as μl. CO_2/min. for 1 ml. serum or 1 ml. of an extract of 8 mg. acetone-dried powder.

Substrate	Enzyme activity (μl. CO_2/ml./min.)	Ratio
A-Type esterase (serum)		
Phenyl acetate	401	$>10^3$
Acetylcholine	<0·4	
B-Type esterase (intestinal mucosa)		
Tributyrin	638	
Butyrylcholine	3·8	$1{\cdot}7 \times 10^2$
A-Type esterase (pancreas)		
Tributyrin	2800	
Butyrylcholine	<0·2	$>1{\cdot}4 \times 10^4$

DISCUSSION

Persistent attempts have been made to characterize the group of enzymes able to hydrolyse esters. The results given in this paper show why attempts using only a variety of substrates have not been generally successful. A-Type esterase in rat pancreas hydrolyses the same triglycerides as the B-type esterase of intestinal mucosa. A method of separating the esterases in sera, previously described by Aldridge (1953*a*), has been shown to be generally applicable to the esterases of tissues. In the work on the esterases of serum, E 600 was the inhibitor chiefly used though TEPP and DFP gave the same results. It is important to consider only the 'irreversible' or 'phosphorylating' type of inhibition (Aldridge, 1953*c*). The results in this paper show that it is unwise to rely on only one inhibitor. For instance, although the A-type esterase of pancreas is not inhibited by TEPP, DFP or mipafox, it is inhibited by 10^{-5}M E 600. However, this inhibition is reversible. Most of the work described has been concerned with characterizing three enzymes which, when separated, are quite dissimilar. The A-type esterase of pancreas readily hydrolyses triglycerides but aromatic esters hardly at all, while for the serum A-type esterase the reverse is true. The B-type esterase of intestinal mucosa hydrolyses both triglycerides and phenyl esters with equal facility. Although these enzymes when separated have very different characteristics much difficulty has been experienced by earlier workers in devising methods for their determination. The Tween compounds (fatty acid esters of polyoxyethylene derivatives of sorbitan) have been used as specific substrates for lipase (Archibald & Ortiz, 1946; Boissonas, 1948; Gomori, 1945, 1949). Recently, however, the use of Tween 20 (laurate) has been recommended for the determination of esterase (Copenhaver, Stafford & McShan, 1950). Nachlas & Seligman (1949) have pointed out that the distribution of esterase in the rat, shown histochemically using β-naphthyl acetate, is very similar to that obtained by Gomori (1945) using Tween 20. Using organophosphorus inhibitors it has been shown conclusively that both Tween 80 (oleate) and Tween 20 are hydrolysed both by the intestinal esterase (B-type) and by pancreatic lipase (A-type). These esterases are extremely difficult to separate by the conventional protein purification methods, and the results above illustrate the difficulty of characterizing them by the use of different substrates. Although tributyrin is often used as a substrate for lipase it is clear that this substrate is quite unsuitable for this purpose. This was pointed out by Cherry & Crandall (1932). The use of organophosphorus compounds will undoubtedly help to characterize the enzymes which have been named after the substrates they hydrolyse, e.g. vitamin A esterase (Ganguly & Deuel, 1953; Krause & Alberghini, 1950; McGugan & Laughland, 1952), cholesterol esterase (Fodor, 1950) aspirin esterase (Vincent & Lagreu, 1949) and esterases hydrolysing indoxyl esters (Barrnet, 1952). That for histochemical methods the specificity conditions must be extremely stringent has been illustrated by Denz (1953) who showed that at the myoneural junction the high concentrations of cholinesterase produced staining when using β-naphthyl acetate as substrate.

Recently Mendel *et al.* (1953) have reported an enzyme in rat brain which is less sensitive to inhibition by E 600 than that of serum (Aldridge, 1953*a*). They have called this enzyme C-esterase in contrast to the B-esterase of serum. It has now been agreed in discussion with Professor Mendel that such differences in sensitivity do not warrant a separate classification. Accordingly, the esterase of Mendel *et al.* (1953) will be classified as a B-type esterase. The esterases examined in the paper have all been classified into the two groups, A- or B-types.

The substrate patterns of the A-type esterase of pancreas (lipase) and the B-type esterase of intestine (esterase) against triglycerides are similar. The differences between lipase and esterase have never been clearly defined. Now that their activities have been separated using organophosphorus compounds, certain differences may be discussed. Lipase hydrolyses triolein much more readily than esterase and esterase hydrolyses aromatic esters more readily than lipase. The effects of emulsification of the substrates on the activity of both enzymes provides a clue to a more fundamental difference. Desnuelle (1951), in a discussion of this question, has asked 'Et ne faudrait-il pas simplement baser la distinction entre substrat esterasique et substrat lipasique sur la solubilite dans l'eau?'. A comparison of the activity of lipase and esterase against tributyrin under identical conditions shows that only the activity of lipase is increased by emulsification of the substrate. The activity of esterase is the same whether the substrate is emulsified or added as drops from a Pasteur pipette. The replacement of hydrolysed substrate to the aqueous phase cannot be a rate-determining factor. Schønheyder & Volqvartz (1945*b*) have also shown that the affinity of pig pancreas for tricaproin is very low in homogeneous solution. With increasing amounts, as soon as a heterogeneous system is produced, there is a large increase in reaction velocity which rapidly reaches a maximum with increasing surface area of the substrate. It is suggested that esterase acts on the substrate in solution in the aqueous phase while lipase has the ability to catalyse the hydrolysis of the substrate at the oil-water interface. On this basis esterase will not be able to hydrolyse triolein because it is so insoluble, but it should be able to hydrolyse an ester of a higher fatty acid if it were in solution. Tween 80 (oleate) is such an ester and it has been shown that relative to tributyrin, esterase hydrolyses it more readily than lipase. The locations where these enzymes exert their activity in a physiological sense does not rule out such an idea.

Using the information gained by studies such as those described on a particular tissue, organophosphorus compounds might well provide useful tools to examine physiological problems. For instance there are the rival theories of Frazer (1948) and Verzár (1948) for the mechanism of fat absorption. Recent work, using the techniques of partition chromatography and radioactive isotopes, has shown that after ingestion of free fatty acids triglycerides are found in the lymph (Borgstrom, 1952*a*). If triglycerides containing labelled glycerol are ingested then the triglycerides recovered from the lymph contain much unlabelled glycerol (Reiser, Bryson, Carr & Kuiken, 1952). Further, if monoglycerides are ingested containing labelled fat and labelled glycerol, triglycerides are recovered from the lymph and the relative activities of their fatty acid and glycerol contents indicates that there must have been considerable hydrolysis of the monoglycerides before resynthesis (Reiser & Williams, 1953). Monoglycerides are resistant to hydrolysis by lipase (Borgstrom, 1952*b*, 1953), and it is therefore clear that considerable chemical activity is taking place between the gut and the lymph. This suggests the need for an examination of the necessity of esterase and for lipase in these processes. It would be relatively easy to maintain animals with their intestinal esterase at a low level by feeding with low concentrations of an organophosphorus compound. Di-*iso*propyl *p*-nitrophenylphosphate would appear to be a suitable compound. It is stable, has an extremely high activity against B-type esterase, and is at least 1000 times less active against true cholinesterase (Aldridge, 1953*d*).

SUMMARY

1. With the method of using organophosphorus compounds for the differentiation of esterases into A- and B-types, another A-type esterase in pancreas of the rat has been demonstrated.

2. The properties of the A-type esterases from serum and pancreas and the B-type esterase of intestinal mucosa have been examined.

3. From a comparison of the A-type esterase of pancreas (lipase) and the B-type of intestinal mucosa, suggestions have been discussed on the difference between esterase and lipase.

4. The uses of organophosphorus compounds for the characterization of esterases have been discussed.

I am grateful to Dr H. Coates (Albright and Wilson Ltd.), and Dr G. S. Hartley (Pest Control Ltd.), for various samples of organophosphorus compounds used in this work. I am also grateful to Dr K. K. Cheng for obtaining the specimens of rat pancreatic juice and to Miss J. E. Cremer for technical assistance.

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A Study of Some Simple Peptide Complexes with Zinc and Cadmium Ions in Aqueous Solution

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Previous studies (Perkins, 1952, 1953*a*) of metal complexes in aqueous solution were restricted to amino acids. It has already been shown (Monk, 1951*a–c*; Perkins, 1952) that for the simplest peptide, glycylglycine, complexes are formed which have smaller stability constants than those of glycine. Dobbie, Kermack & Lees (1951) studied the copper complexes of glycylglycine and diglycylglycine and found that the effect of the second peptide bond was to further reduce the stability constant. In this paper a range of simple peptide complexes with zinc and cadmium ions are examined. Some of the results have been the subject of a preliminary communication (Perkins, 1953*b*).

EXPERIMENTAL

Materials. The following AnalaR (British Drug Houses Ltd., Poole) salts were used in 0·01 M aqueous solution: $ZnSO_4$, $7H_2O$ and $3CdSO_4$, $8H_2O$. All the peptides were vacuum-dried over P_2O_5 at 100° for 48 hr., weighed and made up in 0·02 M solution. The peptides and complexes of glycyl-L-tryptophan and glycyl-L-tyrosine were relatively insoluble, and were therefore used in 0·004 M and 0·002 M solutions respectively. All the peptides were obtained from Roche Products Ltd. unless otherwise stated. The sample of triglycylglycine was kindly given by Dr Hanby, Courtaulds Ltd., Maidenhead. The peptides, glycyl-DL-serine (Fischer & Roesner, 1910), glycyl-DL-phenylalanine (Fischer & Schoeller, 1907), DL-alanyl-DL-alanine (Fischer & Raske, 1906) and glycyl-DL-isoleucine (Abderhalden, Hirsch & Schuler, 1909) were synthesized by the methods cited.

The purity of the peptides was checked by two-dimensional filter paper chromatography and nitrogen content using the micro-Kjeldahl procedure.

Potentiometric titration. A Cambridge Instrument Co. Ltd. bench type pH meter was used with a glass electrode and saturated calomel half-cell. The titrations were carried out as outlined by Albert (1950) with minor modifications. The dissociation constant for the α-amino group was determined at 25° under the conditions subsequently used for the titrations in the presence of the metal. These titrations were made on solutions in which the ratio of acid to metal was 2:1. For most titrations equal volumes of stock metal and amino acid solutions were mixed and then stirred with O_2-free N_2 for 10 min. At the mid-point of the titration the volume was 50 ml. All measurements were made at 25·0±0·1° in a thermostatically controlled water bath.

Calculations. The system to be considered in calculating the stability of each complex is

$$M^{2+} + \text{Peptide} \rightleftharpoons \text{Complex } I^{+} \text{ (association constant} = K_1).$$

The calculation of log K_1 takes account of the proportion of the peptide in the form available for chelation at a particular pH. This form of the peptide is that in which the carboxyl group is ionized and the amino group un-ionized. The method of calculating values for log K_1 for each point on the titration curve was that of Albert (1950).

RESULTS

The stability constants of the peptide complexes are shown in Table 1.

Table 1. *The stability constants at 25° for the zinc and cadmium complexes of a range of peptides*

Peptide	pK	Stability constant: Zn log K_1	Stability constant: Cd log K_1
Glycine	9·73	5·0	4·4
Glycylglycine	8·21	3·6	3·2
Diglycylglycine	8·02	2·6	2·0
Triglycylglycine	7·91	2·9	2·8
DL-Alanine	9·90	4·9	4·2
DL-Alanyl-DL-alanine	8·51	*	3·0
DL-Alanylglycine	8·17	3·00	2·6
Glycyl-DL-alanine	8·17	4·1	3·6
Glycyl-L-leucine	8·13	3·6	3·1
L-Leucylglycine	7·96	3·1	1·9
L-Leucylglycylglycine	7·66	1·8	1·5
Glycyl-DL-serine	8·34	3·7	3·3
Glycyl-DL-phenylalanine	8·28	3·8	3·3
Glycyl-L-tyrosine	8·22	2·6	2·2
Glycyl-L-tryptophan	8·04	3·3	2·7
Glycyl-DL-isoleucine	7·96	3·8	3·4

* Insoluble complex formed.

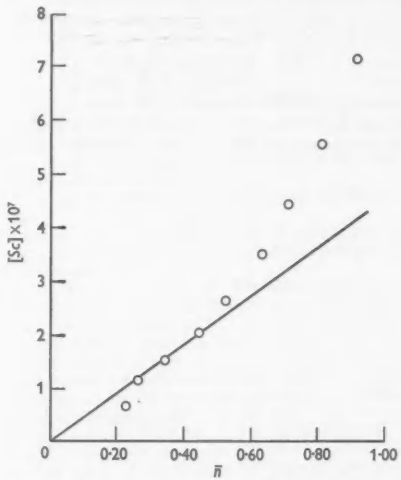

Fig. 1. A plot of the concentration [*Sc*] of histidine available for chelation against the proportion ($\bar{n}$) of the zinc complex formed. The line represents the theoretical result.

DISCUSSION

In the calculation of log K_1 values not all points on the titration curve are suitable. Albert (1950) restricted his calculations to that part of the curve where between 10 and 70 % of the 1:1 metal amino acid (or peptide) complex was formed. Below 10 % it is difficult to obtain reliable values as small errors in the measurement of the volume of added KOH make a considerable difference to the pH observed. Above 70 % formation of the 1:1 complex there is an increasing tendency to form the 1:2 complex. This produces a drift in log K_1 values as shown in Fig. 1, which is a plot of $\bar{n}$ (the proportion formation of the 1:1 complex) against [*Sc*] (the concentration of peptide available for chelation) for zinc and histidine. The curve remains linear to approximately 50 % formation of the 1:1 complex. The deviation beyond this point arises from neglecting the formation of the 1:2 complex in the calculations (Albert, 1950). The value for log K_1 calculated from the position of 70 % complex formation does not, however, vary by more than 0·23 unit from the mean value obtained from the 10–50 % range. The example shown above is an extreme case as, unlike the peptide experiments, two ionizing groups have to be titrated because the zinc ions react with both of them. This produces a dilution effect, which has not been corrected for, and a further source of error in that two pK values are used in the calculations. The individual peptide log K_1 values averaged were within the range ± 0·15 unit.

An attempt was made to determine K_1 values of the cobalt glycylglycine complexes by measurement of colour intensity. This experiment failed because the intense colour measured was not a property of the simple complex but one of an oxygen-containing derivative (Gilbert, Otey & Price, 1951).

In Table 1 the constants for the complexes of composition 1 metal:2 peptide are not recorded as these complexes were mostly insoluble.

A comparison of the stability constants of the peptides and their constituent amino acids shows that there is a considerable decrease on peptide formation. This is not only shown by the homologous glycine and α-alanine series but also by the peptides in which two different amino acids are united.

The length of peptide chain. The progressive lengthening of the peptide chain from glycine to diglycylglycine and α-alanine to alanylalanine shows for the 1:1 complexes that the largest decrease in stability constants occurs when the first and second peptide bonds are introduced. Dobbie *et al.* (1951) found the same effect for the copper complexes of glycylglycine and diglycylglycine. The series leucylglycine and leucylglycylglycine shows

the same effect. In one tetrapeptide examined, the introduction of the third peptide bond produced a small increase in stability constants.

The effect of introducing other amino acid residues, which are aliphatic homologues of glycine, is shown to be small by the study of alanylglycine, glycylalanine, leucylglycine and glycylleucine. When, however, the residues were reversed in order in each peptide a considerable alteration in stability constants was observed.

Peptides of glycine. A comparison between the stability constants of glycylglycine and a range of glycyl peptides is shown in Table 1. In each example the effect of the peptide bond is found to be that of decreasing the stability constants below those of the parent amino acids. The same order was followed, within experimental error, for the stability constants for these complexes, as was found for the amino acids forming the second residue (Perkins, 1952, 1953*a*). This means that it may be possible to predict roughly the order of stability constants for a simple peptide on the basis of its composition.

Smith (1953) discussed the mode of action of manganese as an activator of dipeptidase and suggested that a coordination complex was formed between the metal and the substrate. He found that zinc, cadmium and silver ions were inhibitors for the enzyme. Each of these metals forms complexes with glycylglycine with larger log K_1 values than that for manganese (Monk, 1951*a*–*c*) and would therefore be expected to act as inhibitors if a substrate–metal complex is formed. It is in biological systems of this nature that a knowledge of the relative stability constants may become of importance.

SUMMARY

1. Zinc and cadmium complexes of peptides have lower stability constants than the corresponding complexes of free amino acids.

2. Examination of the homologous peptide series glycine to triglycylglycine shows that complex stabilities decrease further with the introduction of the first and second peptide bonds but increase slightly with the third.

3. The order in which the amino acid residues of the peptide occur is shown to be important.

4. The glycyl peptides of a number of amino acids have complex stability constants which can be roughly predicted from their amino acid content.

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